THE HIGH AFFINITY CALCIUM BINDING SITES IN FIBRINOGEN

Joan Ross

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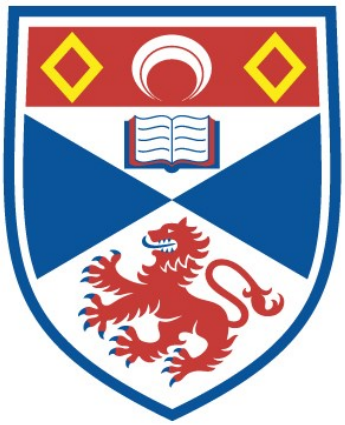

1983

THE HIGH AFFINITY CALCIUM BINDING SITES IN FIBRINOGEN

being a thesis presented by

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to the University of St. Andrews

in application for the degree of

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ABSTRACT

A purification method to obtain a fibrinogen preparation with a high proportion of intact molecules and free from contaminating plasminogen and Factor XIII was developed. Such fibrinogen preparations were used in cross-linking and plasmin-digestion studies to investigate the possible involvement of the carboxyl terminal regions of the Aα-chain in a high affinity calcium-binding site. The results of these investigations gave no evidence to support such a role for the carboxyl terminals of the Aα- chains.

Highly intact fibrinogen preparations were also used in experiments to re-assess the number of high affinity calcium-binding sites in the molecule. The technique of flow dialysis was employed. Results from these experiments were in agreement with previously published data that there are three high affinity calcium-binding sites in fibrinogen. Indirect evidence was obtained which suggests that the chelator EGTA binds to the fibrinogen molecule.

DECLARATION

I hereby declare that the following thesis is based on work carried out by me, that the thesis is my own composition, and that no part of it has been presented previously for a higher degree.

This research was carried out in the Department of Biochemistry in the United College of St. Salvator and St. Leonard, The University of St. Andrews, under the supervision of Dr. G.D. Kemp.

Joan Ross

CERTIFICATE

I hereby certify that Joan Ross has spent nine terms engaged in research work under my direction and she has fulfilled the conditions of Ordinance General No.12, and Resolution of the University Court, 1967, No. 1, and that she is qualified to submit the accompanying Thesis for the degree of Doctor of Philosophy.

Dr. G.D. Kemp

ACKNOWLEDGEMENTS

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ACADEMIC RECORD

I first matriculated at the University of St. Andrews in October, 1974 and graduated with the degree of Bachelor of Science, Upper Second Class Honours in Biochemistry in July 1978.

I matriculated as a research student in the Department of Biochemistry, University of St. Andrews in October 1978.

C O N T E N T S

ABBREVIATIONS

AAS:	Atomic Absorption Spectrometry
DMS:	dimethyl subermidate dihydrochloride
EDTA:	ethylenediaminetetra-acetate
EGTA	ethyleneglycolbis(amino-ethylether)tetra-acetate
PAGE:	polyacrylamide gel electrophoresis
PAGs:	polyacrylamide gels
POPOP:	1,4-Di-2-(5-Phenyloxazolyl)benzene
PPO:	2,5-Diphenyloxazole
SDS:	sodium dodecyl sulphate

INTRODUCTION

1.1. CHEMICAL COMPOSITION OF FIBRINOGEN

Fibrinogen is a soluble protein, with a concentration in human plasma of about 3 mg/ml, whose main biological function is as a precursor to fibrin the major structural element in the haemostatic plug. It may also play a significant role in the defence mechanisms against bacterial infection (Hawiger et al., 1975), the inflammatory response (Bernhart et al., 1971) and in wound healing (Duchart & Beck, 1968). However it is because of fibrinogen's importance in haemostasis and thrombo-embolic disease that its structure and the mechanism by which it is converted to fibrin have been so intensively studied.

Human fibrinogen is a dimer (Blomback & Yamashina, 1958) the two identical halves each consisting of three polypeptide chains. These chains have been designated $A\alpha$, $B\beta$ and γ and have molecular weights of 67,000 56,000 and 47,000 respectively (Gaffney & Dobos, 1971). This gives a value of 170,000 for the molecular weight of the half molecule and 340,000 for the whole molecule which is in agreement with the molecular weight values obtained from sedimentation data in the analytical centrifuge (Shulman, 1953; Caspary & Kekwick, 1957) and by light scattering studies (Katz et al., 1952). 4-5% of the molecular weight is contributed by covalently bound carbohydrate and there is also a small amount of ester-linked phosphoric and sulphuric acid. There are 56-58 cysteine residues in the fibrinogen molecule and all are involved in disulphide bonding (Loewy et al., 1961). The three polypeptide chains in each

half-molecule are held together by disulphide bonds (Clegg & Bailey, 1962; Henschen, 1963) and the two half molecules are linked by disulphide bridges between the two Aα - chains and the two γ- chains (Blomback, 1970).

1.2. FIBRINOGEN DEGRADATION PRODUCTS

Fibrinogen is the principal substrate for two plasma enzymes plasmin (EC 3.4.4.14) and thrombin (EC 3.4.4.13). Fibrinogen is converted to the insoluble polymeric gel fibrin, by the action of thrombin which cleaves small peptides, the so-called fibrinopeptides, from the amino terminal ends of the Aα- and Bβ - chains (Bailey et al., 1951; Lorand, 1952). The resulting fibrin monomers spontaneously polymerise to a fibrin gel which is stabilised by the action of a transglutaminase, Factor XIII, which catalyses the formation of covalent bonds between α- chain pairs and γ-chain pairs in adjacent molecules (McKee et al., 1970; Chen & Doolittle, 1971). The initial action of thrombin is on fibrinopeptide A which is removed rapidly from the Aα - chain. Fibrinopeptide B is released after a lag phase during which polymerisation has alredy commenced (Blomback et al., 1957; Blomback & Vestermark, 1958; Teger-Nilsson, 1967). Blomback et al. (1957) have shown that fibrinopeptide A release is sufficient for gelation to occur without any detectable release of fibrinopeptide B.

In vivo, plasmin is primarily responsible for the dissolution of the fibrin clot. In vitro it has been utilised along with cyanogen bromide (CNBr) to help characterise the primary structure of fib-

rinogen and elucidate the gross structure of the molecule. Plasmin is the active form of the enzyme plasminogen and the first detailed study of the products of fibrinogen-plasmin interactions was made by Nussenzweig et al. (1961). They concluded that the terminal degradation products D and E account for 70% of the fibrinogen molecule. A general scheme for the digestion of fibrinogen by plasmin has been proposed by Marder et al.(1969), in which fibrinogen is cleaved asymmetrically. This scheme has been elaborated upon but the major aspects of the degradation sequence are generally accepted (Figure 1). The individual polypeptide chains vary in their susceptibility to plasmin digestion, the Aα-chain being more readily digested than Bβ with the γ-chain least susceptible to digestion (Murano et al., 1972). The first step in digestion involves the removal of the carboxyl half of the Aα-chains leaving fragment X. This is followed by the loss of the amino-terminal end of the Bβ-chains and the asymmetrical splitting of the three chains in one half of the molecule releasing one fragment D. The remaining fragment is fragment Y and subsequent lysis releases another D fragment leaving the other core fragment - fragment E. Thus the fibrinogen molecule gives rise to two fragments D and one fragment E. Fragment E is dimeric, containing fragments of all six chains held together by disulphide bonds, and corresponds to the N-terminal region of fibrinogen while fragments D arise from regions located near the carboxyl terminal of the fibrinogen molecule. In vitro digestion of

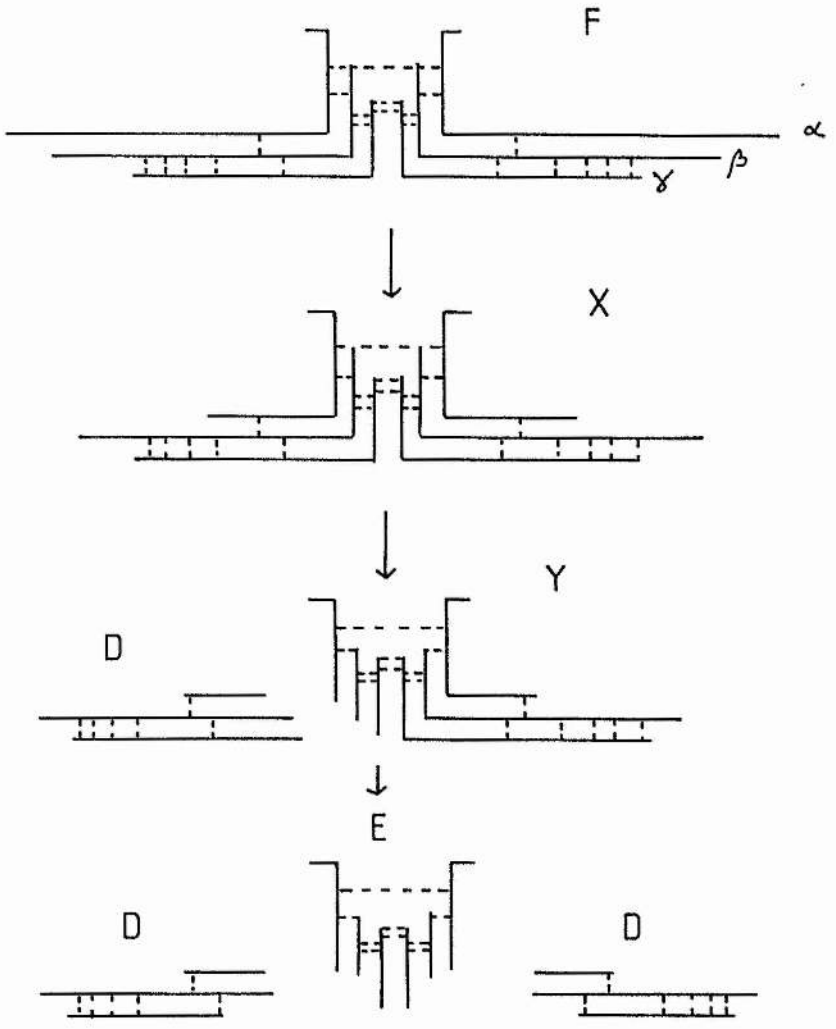

Figure 1: Schematic diagram of the digestion of fibrinogen (F) to its core fragments D and E by the action of plasmin (Latallo, 1973)

fibrinogen by plasmin results in a family of D fragments which are heterogeneous in both size and charge with molecular weights ranging from 73,000 to 100,000 (Gaffney & Dobos,1971). This heterogeneity arises from varying degrees of digestion of the carboxyl region of the γ-chain (Ferguson et al., 1975) and is influenced by the presence of calcium ions or chelating agents such as EDTA (Purves et al., 1978a).

Fibrinogen contains about 60 methionine residues and cyanogen bromide reacts at these residues resulting in some 30 fragments. Some are single polypeptide chains while others consist of a number of peptide fragments held together by disulphide bridges. Those fragments containing disulphide bonds are called disulphide knots (DSK). The majority of the cysteine residues are contained in four fragments of which two are hydrophobic and two are hydrophilic in nature. The largest CNBr fragment, of molecular weight 60,000, is composed of the N-terminal fragments of all six polypeptide chains of fibrinogen (Blomback et al., 1968) and is known as the N-terminal disulphide knot (N-DSK). It contains 11 of the 28 disulphide bridges of fibrinogen and has a considerable portion of its structure in common with fragment E. Fragment E is smaller lacking the first 53 amino acids of the $B\beta$-chain and a carboxyl portion of the γ-chain when compared to the N-DSK but has longer $A\alpha$- and $B\beta$-chains. The other hydropholic DSK corresponds to the carboxyl end of the $A\alpha$-chain while the two hydrophobic knots are contained within plasmin fragment D. The chain remnant composition of these fragments may be of importance when considering the 3-dimensional shape of the

fibrinogen molecule and its behaviour in solution.

1.3. HETEROGENEITY

Fibrinogen heterogeneity has been characterised by differences in solubility (Mosesson & Sherry, 1966; Lipinska et al., 1974) electrophoretic mobility (Takagi & Iwanaga, 1969; Mosesson et al., 1974; Gaffney, 1971; Arnesen, 1974) molecular weight (Mosesson et al., 1967; Mosesson et al.,1972b) and chromatographic elution profiles (Finlayson & Mosesson, 1963; Finlayson & Mosesson, 1964; Mosesson & Sherry, 1966). This heterogeneity can, in part, be explained by differences in carbohydrate content of the polypeptide chains (Finlayson & Mosesson, 1963; Mosesson & Sherry, 1966) variations in incorporation of phosphate (Blomback et al., 1963; Blomback et al., 1972) and micro-heterogeneity in the amino acid composition of the molecule (Gerbeck et al., 1969).

Amino acid variation is responsible for the presence of two distinct γ-chains, found in both human (Mosesson et al.,1972a) and bovine fibrinogens (Mosher & Blout, 1973), which are separated by anionic exchange and alkaline polyacrylamide gel electrophoresis. Some of this heterogeneity may reflect individual gene differences as Gaffney (1971) has reported finding less heterogeneity in single source fibrinogen than in pooled samples.

Heterogeneity of fibrinogen is also reflected in the solubility of the preparation. Mosesson & Sherry (1966), who prepared nine different fibrinogen fractions, all with clottability greater than 90%, by glycine/ethanol precipitation found that different fractions

contained fibrinogen of different molecular weights as determined by analytical ultracentrifugation and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). These differences in solubility are related to differences in molecular weight with low solubility fibrinogen having a higher molecular weight than high solubility preparations (Mosesson et al., 1967). Mosesson et al. (1972b) confirmed this and demonstrated, by tryptic digest peptide maps of the individual polypeptide chains of fibrinogen, Aα, Bβ and γ, that the molecular weight variations shown by solubility and electrophoretic studies arise from the degree of degradation of the Aα-chain only and this degradation is at the carboxyl terminal of the chain. This heterogeneity in the molecular weight of the fibrinogen molecule is thought to be the result of in vivo enzymic degradation (Sherman et al., 1969; Semeraro et al., 1977) probably by plasmin (Mosesson et al., 1974).

Mills and Karpatkin (1970) found, using SDS-PAGE, two Aα-chain species differing in molecular weight by 3,000. This molecular weight difference corresponds to the loss of a 27-residue peptide released during the early stages of plasmin digestion of fibrinogen in vitro (Cottrell & Doolittle, 1976).

Fibrinogen prepared from freshly collected plasma contains both Aα-chain species suggesting that removal of this small peptide from the carboxyl end of the Aα-chains occurs in vivo. Semeraro et al. (1977) have demonstrated that the proportion of the smaller Aα-chain is greater in fibrinogen prepared from outdated human

plasma. Thus molecular weight heterogeneity of the Aα-chain of fibrinogen, commonly found in commercial preparations, is probably a result of both in vivo and in vitro degradation.

1.4. FIBRINOGEN SHAPE

At present there is no general agreement on the size and shape of fibrinogen despite the recent success of the completion of the sequencing of the human fibrinogen molecule (Henschen & Lottspeich, 1977; Henschen et al., 1978; Watt et al., 1979a,b). During the past 30 years many groups have investigated fibrinogen structure. To date, unmodified fibrinogen has not been crystallised in a form suitable for X-ray diffraction experiments therefore information on the size and shape of the molecule has been derived mainly from physico-chemical data and electron microscopy.

Electron microscopy has given rise to four principal models. Hall & Slayter (1959) used shadow casting to study bovine fibrinogen. This technique requires dehydration of the molecule and under these conditions the molecule appears as a linear array of three nodules held together by thin threads (Figure 2a). The two outer nodules appear to be identical and slightly larger than the central nodule. The overall length of the molecule was estimated as 47.5 nm $\pm$ 2.5 nm. This model has been supported by the work of Fowler & Erickson (1979) and Price et al. (1981) the former using both shadowing and negative staining techniques. In both these studies the tri-nodular rod was found to have a degree of flexibility with both crescent and S-shaped molecules visible. Recent studies employing antibodies (Telford et al.

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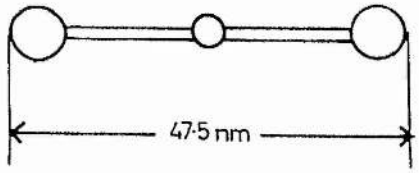

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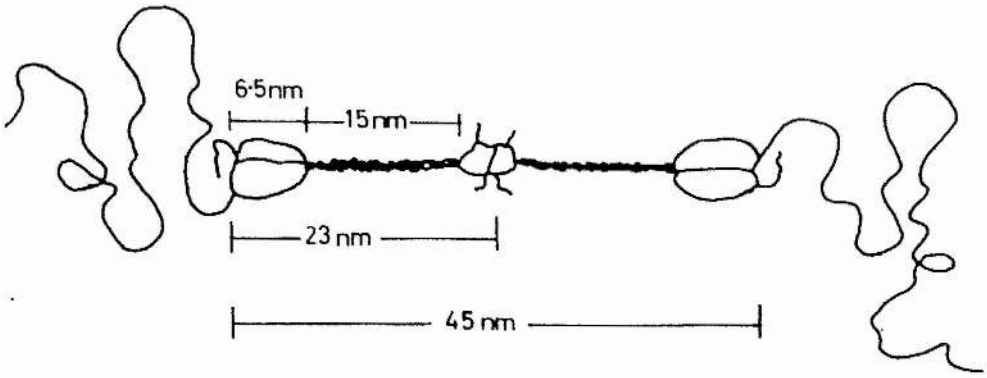

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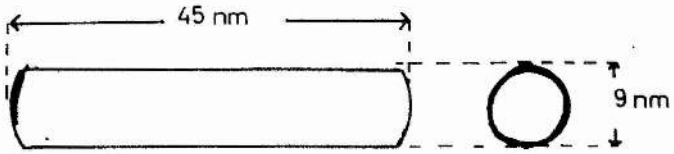


Figure 2: Proposed Shapes for the Fibrinogen Molecule

a) Hall & Slayter (1959)

b) Doolittle et al. (1977)

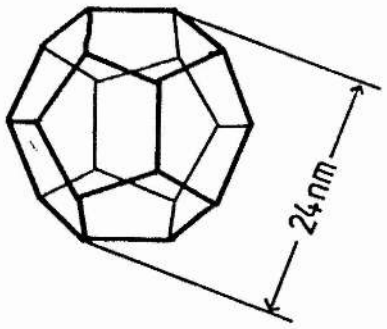
c) Bachmann et. al. (1975)

1980) and antibody fragments (Price et al., 1981) have identified the centre nodule as corresponding to Fragment E and the terminal nodules as analagous to fragment D. Doolittle et al. (1977), using computer simulation techniques, found support for the Hall & Slayter tri-nodular model from primary structure data and indicated plasmin resistant and plasmin susceptible sites in the molecule (Figure 2b). Coiled coils of the three polypeptide chains are thought to link the three domains while the fibrinopeptides and carboxyl terminal regions of the Aα-chains swim free and are, therefore, more susceptible to enzyme attack. This is in agreement with the results of Fowler et al. (1980) who investigated by electron microscopy the plasmin digestion products of fibrinogen. They found fragment X to be indistinguishable from fibrinogen, fragment Y to be two linked nodules and the terminal products D and E single nodules.

Bachmann et al.(1975) studied the hydrated molecule by the freeze-etching technique and found molecules which appeared as cylinders with rounded ends 45 nm $\pm$ 1.5 nm long and diameter 9 nm $\pm$ 1 nm (Figure 2c), dimensions compatible with the Hall & Slayter model. These molecules also showed some flexibility and the water of hydration was calculated as 4 g H_2O/g protein.

Koppel (1966, 1970) employed another electron micrograph technique, that of negative staining. He described spherical particles of 24 nm diameter which, he suggested, had the form of a pentagonal dodecahedron (Figure 3a). From these results for the hydrated molecule he proposed that fibrinogen is a non-compact particle

a)



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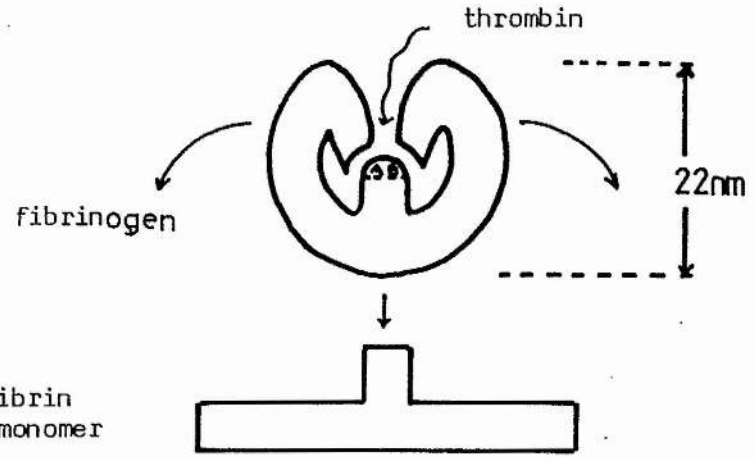


Figure 3: Proposed shapes for the Fibrinogen Molecule

a) Koppel (1966)

b) Hudry-Clergeon et. al. (1975)

with the polypeptide chains situated on the surface of the molecule along the edges of the dodecahedron and the interior filled with solvent.

Pouit et al. (1972), also using negative staining, found spherical particles with dimensions in agreement with Koppel's data. They extended the model by suggesting that, on the action of thrombin, there is a conformational change in the molecule, as the fibrinopeptides are released, giving a linear arrangement of the fibrin monomer (Figure 3b)(Hudry-Clergeon et al. 1975; Pouit et al. 1975).

This variability of results has, in part, been attributed to an abnormally high degree of hydration of the fibrinogen molecule (Bang 1964: Karkow et al., 1972). There also appears to be some correlation between the technique used and the molecule shape proposed with shadow casting favouring clyindrical molecules composed of nodules and negative staining giving rise to spherical particles.

More recently however Fowler & Erickson (1979) obtained trinodular structures using both shadowing and negative-staining techniques. Estis & Haschemeyer (1980) have prepared negatively-stained micrographs at varying concentrations of fibrinogen and found rod-shaped molecules at low protein concentrations and results similar to Koppel's at high protein concentrations, suggesting that the latter results are artefacts. Conversely, Mosesson et al. (1979) carried out shadow casting of fibrinogen at varying protein concentrations and found that, at low con-

centrations, particles of 10 nm diameter equivalent to single Hall & Slayter nodules predominated. This work is in agreement with that of Blakey et al. (1977).

Similarly physico-chemical studies have given rise to a number of possible diverse shapes for the fibrinogen molecule. Hydrodynamic data indicates a rod-shaped molecule (Scheraga & Laskowski, 1957) however the interpretation of hydrodynamic data is dependent on the estimation of the degree of hydration of the molecule. A swollen lattice structure is also compatible with the data if a higher value, of 6 g H_2O/g protein, is assumed for the degree of hydration of the molecule (Marguerie & Struhrmann, 1976). Serrallach et al. (1979) were unable to obtain good agreement between experimentally-derived hydrodynamic parameters and those calculated for the various fibrinogen shapes prepared from electron micrograph data even when the degree of hydration was varied within wide limits to a maximum of 9 g H_2O/g protein. Agreement could only be obtained if a highly flexible molecule of length 90 nm, corresponding to two Hall & Slayter molecules joined together end to end, and 9 nm diameter was envisaged. Marguerie (1979), using small-angle neutron scattering, proposed a flattened disk with central cleft, which has some degree of flexibility. Lederer (1979) also suggested a flexible molecule from data obtained from small-angle X-ray scattering but visualised a clyindrical molecule of length 45 nm.

Intact fibrinogen has not been crystallised and, therefore, X-ray diffraction studies have not been performed. Weisel et al. (1978) have carried out X-ray diffraction studies on fibrinogen modified by bacterial proteases. As a result of these investigations the authors concluded that fibrinogen is a linear molecule of length 45 nm and is closely related in structure to fibrin. Such work must be interpreted with care however as the conversion of fibrinogen to fibrin is a result of only a small change in the molecular weight of the molecule.

1.5. CROSS-LINKING

Chemical cross-linking offers an alternative method for studying the shape of the fibrinogen molecule. Chemical cross-linking agents cross-link amino-acid residues which lie a definite distance apart. The position of each cross-link is dependent on the tertiary structure of the protein and may identify regions of the molecule which are in close proximity. Bifunctional agents, therefore, have the potential to provide information about changes in conformation brought about by enzymic digestion or physico-chemical treatments.

The results of work by Furlan & Beck (1975), who employed the bifunctional agent Tetranitromethane to cross-link fibrinogen, indicate that the Aα- chain is on the surface of the molecule. This is compatible with the known susceptibility of the Aα-chain

to plasmin digestion.

1.6. CALCIUM BINDING

The importance of calcium in the blood-clotting process has long been recognised but it is only recently that the importance of calcium ions to the structural integrity and function of fibrinogen have been emphasised. Fibrinogen preparations are commonly contaminated with Factor XIII and traces of prothrombin whose actions are readily inhibited by the removal of calcium ions. Hence the importance of calcium ions as an integral part of the fibrinogen molecule has been overlooked.

Calcium ions bind to fibrinogen (Marguerie et al., 1977), stabilise it against denaturation by acid (Marguerie, 1977), heat and alkali (Ly& Godal, 1972) and limit the digestion of both fibrinogen and fibrin by proteases such as trypsin (Komenko & Belitser, 1963) and plasmin (Haverkate & Timan, 1977).

The plasmin digestion products of fibrinogen prepared in calcium depleted solutions are known to be unstable. Fragment D prepared under these conditions is found to be heterogeneous with a molecular weight range of 80,000-93,000 (Haverkate & Timan, 1977). This heterogeneity is a result of the progressive digestion of the carboxyl terminal of the γ-chain in fragment D. Haverkate & Timan (1977), followed the digestion of fibrinogen by plasmin at physiological concentrations of calcium and reported that the rate of digestion is reduced and a single species of frament D of molecular weight 93,000 results. This is supported by Purves et al. (1978a)

who suggested that calcium ions protect fragment D by binding with high affinity to the γ-chain. They found that the γ-chain, molecular weight 38,000 was not susceptible to further degradation by plasmin while calcium ions were present in solution. But if fragment D was dialysed for long periods against calcium-free solutions or if chelating agents such as EDTA were added, the γ-chain degraded rapidly to a chain of molecular weight 25,000. This digestion of the γ-chain accounts for the appearance of the smaller fragment D of molecular weight 80,000 and fragments D of intermediate size. Calcium ions also lend stability, in the presence of high concentrations of plasmin, to D-dimer a product of plasmin digestion of the fibrin clot (Purves et al. 1978a). Ferguson et al. (1975) suggested that D-dimer was protected by virtue of the Factor XIII cross-linked sites being occupied. However Purves et al. (1978a) have shown that it is the presence of calcium ions,not occupation of the cross-link site,which is responsible for protecting D-dimer as stability is reduced in the presence of chelating agents. The effect of chelating agents is reversible as the re-addition of calcium stabilises the remaining D-dimer and D-species of all types.

Many studies of calcium binding have been made indirectly by studying the effect of the chelator EDTA. In this way Godal (1960, 1969) investigated the influence of calcium on the clotting process. He found an increase in the thrombin clotting time as the EDTA concentration was increased and this effect was through the fibrin polymerisation step rather than the activation of fibrinogen

by thrombin. Furthermore the effect of EDTA was thought to be through the removal of strongly bound calcium ions from the fibrinogen molecule rather than as a direct interaction between EDTA and fibrinogen as suggested by Bithell (1964). However it is generally accepted that as a result of the removal of strongly bound calcium ions the fibrinogen molecule becomes more easily denatured. As a consequence chelating agents such as EDTA must be used with care to avoid irreversible changes in the fibrinogen molecule.

Endres & Sheraga (1972) were first to suggest that there are at least two types of calcium-binding sites in fibrinogen. Maguerie (1977) investigated the calcium-binding properties of bovine fibrinogen and from equilibrium dialysis data proposed three high affinity calcium-binding sites ($K_d 10^{-5}$M) and a number of binding sites, around 12-15, of lower affinity ($K_d 10^{-3}$M). The low affinity binding sites are eliminated in the presence of magnesium ions suggesting that they are non-specific binding sites. However magnesium ions cannot substitute for calcium ions at the high affinity binding sites. Marguerie found two of the three sites to be identical while the third is different being eliminated at pH values below pH 5.0. This suggested that histidine residues were involved in binding calcium through a chelate system at the third site. Lindsey et al. (1978) and Nieuwenhuizen et al. (1979) also investigated the high affinity calcium binding sites in fibrinogen and obtained similar results. They located two of the three binding sites one in each plasmin fragment D. Calcium bound only to

fragment D prepared in the presence of calcium (≥1 mM) not to the smaller D obtained from digestion in the presence of EDTA. This would imply that the high affinity calcium ion binding in fragment D protects against further plasmin attack and is located in the carboyxl terminal of the γ-chain remnant in fragment D. This is supported by indirect evidence from Lawrie & Kemp (1979) who found that the mobilities of Fragment D and the γ-chain of fibrinogen, as measured on sodium dodecyl sulphate polyacrylamide gel electrophoresis, is increased in the presence of calcium, an effect which can be explained by postulating an intra-chain loop in the γ-chain stabilised under denaturing conditions by calcium.

The location of the third high affinity calcium binding site is as yet unresolved. Its location in the molecule may be of great significance if there is, as suggested, a total of three binding sites in the fibrinogen molecule. Assuming the symmetry of fibrinogen the third calcium ion must link the two halves of the molecule and perhaps contribute to a more compact molecular structure. One possible location is in plasmin fragment E but no high affinity calcium binding site has been found in fragment E derived from fibrinogen or cross-linked fibrin (Van Ruijen-Vermeer 1978; Lindsey et al. 1978). Marguerie (1977) has shown that, in the presence of calcium ions, the rate of cleavage of the first bonds to be attacked by plasmin is reduced. As it has been demonstrated that the initial point of attack by plasmin on fibrinogen is in

the carboxyl terminal region of the Aα-chain (Pizzo et al. 1972; Mosesson et al. 1972) it is possible that the third high affinity binding site is situated in this region of the molecule. This would require, if molecular symmetry is to be maintained, a single calcium ion bridging the two halves of the fibrinogen molecule. Credo et al. (1978) found that the presence of fibrinogen reduces the requirement of Factor XIII for calcium. The sections of the fibrinogen molecule responsible for this are the midsections of the α- chain (Credo et al. 1981), the part of the fibrinogen molecule where the α-chain cross-link sites are situated (Chen & Doolittle, 1971; Fretto et al.,1977). This leads to the interesting speculation that this area, which is not part of the major plasmin fragments D and E, may contain a tightly bound calcium ion.

The influence of calcium ions on fibrinogen structure and the manner in which this is reflected in the plasmin degradation products has been used to investigate abnormal fibrinogens. Haverkate et al. (1978) have used the different patterns obtained from plasmin digestion in the presence and absence of calcium to examine abnormal fibrinogen for structural deviations while Rupp et al. (1981) have characterised the abnormal fibrinogen Bern I, which gives rise to hypodysfibrinogenaemia, as having defective calcium binding in the D-domain of the γ- chain.

The role of the tightly bound calcium in fibrinogen is not understood. Laudano & Doolittle (1981) have studied the effect calcium has on the role of the N-terminal sequences of the α- and β-chains exposed by the action of thrombin. These sequences interact with

the D-domain of an adjacent molecule and have a fundamental role in the formation of the fibrin clot. They found that the affinity of the amino terminal tetrapeptide of the β-chain of fibrin for fibrinogen dramatically increases in the presence of 2 mM calcium. By comparison there is no significant increase in the affinity of the tripeptide, corresponding to the amino terminal sequence of the fibrin α-chain, in the presence of calcium ions, although the number of binding sites increase. This increase is attributable to the tripeptide binding to the β-chain site. From this it would seem that calcium increases the accessibility of the peptides to the binding site. If this work is considered alongside that of Credo et al. (1978,1981) a case could be made for the high affinity binding sites in fibrinogen to have a role in the clotting process.

1.7. FLOW DIALYSIS

Previous studies of high affinity calcium ion binding to the fibrinogen molecule, be it from bovine (Marguerie et al., 1977) human (Nieuwenhuizen et al., 1979; Lindsey et al., 1978) or rat plasma(Van Ruijen-Vermeer et al., 1978) have been carried out using the technique of equilibrium dialysis (Scatchard, 1948). This method requires dialysis of the protein sample for up to 48 hours which in the case of fibrinogen could lead to degradation. Another possible method for investigating calcium-binding is flow dialysis. This was first described by Colowick & Womack (1969) when they investigated the binding of sugars to yeast hexoses.

Since then it has been used to study a number of different protein/ligand systems including the binding of calcium to bovine Factor X (Yue & Gertler, 1978). The flow cell described by Colowick & Womack (1969) is shown in figure 4. It was not designed specifically for flow dialysis but for rapid removal of small molecules and as a result has a large membrane surface area to upper chamber volume ratio. Feldman (1978) designed a wedge-shaped cell with a lower chamber of very small volume. This has the advantage of a small membrane surface area to upper chamber volume ratio and the very small lower chamber volume gives almost instantanaeous mixing and, therefore, a very short equilibration time.

The flow dialysis method is based on the rapid measurement of the rate of dialysis of radioactive substrate from an enzyme-substrate equilibration mixture where enzyme-substrate may be interpreted as a protein binding reversibly with a small molecule or ion. The method depends on the observation that all factors affecting the rate of dialysis of diffusible materials are constant except the free i.e. unbound concentration of the dialysable species in the upper comparment. Measurement of the radioactivity of the dialysable substance in the effluent is a measure of the concentration of freely dialysable material in the upper chamber.

This procedure has two distinct advantages over equilibrium dialysis, the more traditional method of determining ligand dissociation of large molecules -

a) because the measurement depends on the attainment

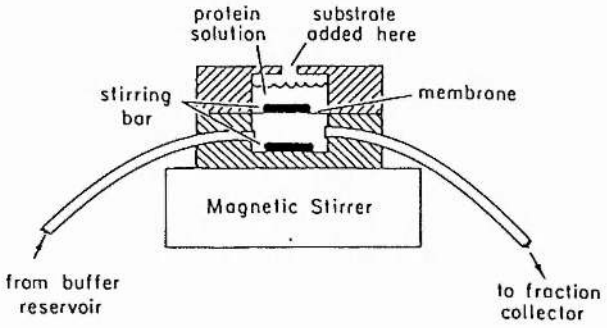


Figure 4: The Flow Dialysis Cell

From: Womack, F.C. and Colowick, S.P. in "Methods in Enzymology" 27, p.465. Ed. by C.H.W. Hirs and S.N. Timasheff (1973) Academic Press.

of chemical equilibrium rather than diffusion equilibrium across the membrane, results may be obtained within minutes and this facilitates the study of labile proteins and proteins with degradable substrates.

b) as such a short time is required and only a small amount of total ligand is removed for any one reading the sample may be used for a series of readings at increasing ligand concentrations.

The initial ligand addition is radioactive but subsequent additions are of unlabelled ligand and by measuring the radioactivity in the effluent buffer sufficient data can be collected from one experiment for a Scatchard plot.

When small metal ions are the binding ligands an alternative way to perform flow dialysis is to utilise chelating agents such as EGTA (Haiech et al., 1980). This overcomes the difficulties of prior removal of the ions from the protein which may result in degradation of the molecule under investigation.

1.8. CALCIUM DETECTION

Both equilibrium and flow dialysis require accurate measurement of the calcium concentrations of the protein starting material and dialysing buffers. A number of methods for calcium determination are available.

Atomic Absorption Spectrophotometry is a highly specific and sensitive method of detection. A suitable absorption band is chosen so that interference from any other element is minimal and using a suitable flame, for calcium a nitrous oxide/acetylene flame is normally employed, calcium concentration to the limit of 10^{-6}-10^{-7}M are detectable. Concentrations of an order of magnitude lower can be dectected if a graphite furnace attachment is used and this recent extension of the technique has the added advantage of requiring only very small volumes of sample for accurate determinations.

Another common method for measuring calcium in solution uses a calcium selective electrode (Simon et al., 1978). This contains an ion exchanger which selectively extracts calcium ions from aqueous solutions. However many ion exchangers, which have a high affinity and therefore a high sensitivity for calcium ions, also show a high affinity for other cations, particularly those of alkaline earth metals such as magnesium, which is a serious disadvantage in fibrinogen studies as buffers containing magnesium are often used. Detection limits are comparable to those obtained by atomic absorption but the electrodes only detect calcium ion concentrations. This could lead to erroneous results when measuring calcium concentrations in protein solutions where the bound calcium may make up a significant proportion of the calcium present.

The use of photoproteins, in particular aequorin, as biological calcium indicators was first suggested by Shimomura et al., (1963).

Aequorin is a bioluminescent protein, isolated from the jellyfish Aequerea, which emits light on the addition of calcium ions thus providing the basis of a quantitive calcium detection system. Although assays with aequorin have lower detection limits of 10^{-7}M photoproteins suffer many of the disadvantages encountered with calcium-sensitive electrodes. Magnesium ions inhibit the calcium-aequorin reaction and in addition the photoprotein solutions are frequently contaminated with EDTA which makes accurate calibration extremely difficult. These agents are best employed to detect low concentrations of calcium ions rather than total calcium concentrations (Blinks et al.,1976) and are not suitable for accurate determination of calcium concentrations in fibrinogen solutions.

A number of metallochrome dyes which are sensitive to calcium are known and their usefulness as indicators is dependent on there being a shift in their absorption spectrum on the binding of calcium. Two of these dyes which are commercially available are murexide and Arsenazo III. Murexide is a very specific indicator commonly used to determine calcium and especially EDTA concentrations (West, 1959) but it lacks sensitivity. Arsenazo III is more sensitive than murexide but it is less specific as it reacts with magnesium. Also, commercial preparations are frequently contaminated with calcium and, as with the calcium selective electrodes and photoproteins, Arsenazo III is best employed to measure calcium ion rather than total calcium concentrations.

1.9. AIM OF STUDY

The role of calcium ions in fibrinogen and their influence on the shape of the molecule are, as yet, poorly understood. Two high affinity calcium binding sites have been located, one in each of the degradation fragments D. These protect against further degradation by plasmin, in vitro, at physiological calcium concentrations. The accepted number of high affinity sites is three and, so far, the third binding site has not been located. A possible location is at the carboxyl terminals of the Aα- chains, and if molecular symmetry is to be maintained, this would require the two Aα- chains to be linked by a single calcium ion. This in turn would probably necessitate a conformational change in the molecule on the conversion of fibrinogen to fibrin. Alternatively, the number of calcium binding sites may have been underestimated through use of heterogeneous fibrinogen preparations which contain molecules with partially degraded Aα- chains.

The original aims of this study were -

i) to develop a rapid purification technique to obtain a fibrinogen preparation with a high proportion of of intact molecules and free from contaminating plasminogen and Factor XIII.

ii) to test the hypothesis, by cross-linking and plasmin digestion studies, that the carboxyl terminal regions of the Aα- chains are involved in calcium binding.

iii) to re-evaluate the number of high affinity calcium binding sites using the flow dialysis technique.

It had been hoped that the N-terminal disulphide knot could be examined as an alternative location for the third calcium binding site. However, because of solubility difficulties this was not pursued.

MATERIALS AND METHODS

2.1. MATERIALS

Human fibrinogen (Grade L) was obtained from KABI Pharmaceuticals, London, U.K. Fresh frozen human plasma was kindly supplied by The Blood Transfusion Service, Ninewells Hospital, Dundee. Benzamidine, Brilliant Blue R, dansylchloride, dimethylsuberimidate hydrochloride, ethyleneglycol bis(-aminoethylether)-N,N'-tetraacetic acid, L-lysine monochloride, N-ethylmaleimide, phenylmethylsulphonylfluoride, bovine serum albumin, cytochrome C, myoglobin, ovalbumin and thrombin were all purchased from Sigma (London) Chemical Co., London, U.K. Acrylamide and methylene-bis-acrylamide were obtained from BDH, Poole, U.K., and were already specially purified for gel electrophoresis. β-mercaptoethanol, calcium chloride hexahydrate, ethylenediamine tetraacetic acid, N,N,N',N'-tetramethylenediamine, sodium dodecyl sulphate Tris(hydroxymethyl)aminomethane, TritonX-100(scintillation grade) and urea were also obtained for BDH. Sepharose 6B and Sephadex G-25 were obtained from Pharmacia Fine Chemicals, Upsala, Sweden. DEAE Cellulose (DE 52) was purchased from Whatman Biochemicals Ltd., Maidstone, Kent, U.K. Trasylol was purchased from Bayer Pharmaceuticals, Surbiton Surrey, U.K. Streptokinase was supplied by Hoechst Pharmaceuticals, Germany. Antisera were obtained from Seward Laboratory, London U.K. $^{45}CaCl_2$(sp. radioactivity 10-40 mCi/mg of Ca) was purchased from the Radiochemical Centre, Amersham U.K. Scintillation fluid (Pico Fluor) was obtained from Packard Instrument Ltd., Caversham, U.K. Chelex-100 was a kind gift from Prof. M. Furlan, Berne, Switzerland.

All other reagents were of analytical grade unless otherwise stated.

2.2. PREPARATIVE METHODS

2.2.1. Lysine-Sepharose

Lysine-sepharose was prepared by the method of Chibber et al. (1974). To 100 g Sepharose 6B were added 250 ml 5% cyanogen bromide (w/v). pH was maintained at pH 11 by the addition of 5 M NaOH and the temperature was kept between 10-20°C by the addition of ice. The reaction was allowed to proceed for 10 min before being stopped by washing the sepharose with 2 L ice-cold $NaHCO_3$. 75 ml of 40% lysine (L-lysine monochloride) solution (w/v), adjusted to pH 8.9 with NaOH, were added and the solution stirred overnight at 4°C. An excess of amino groups, in the form of glycine, was then added and stirring continued for 3-4 hours. The Sepharose was then washed with 1 L 1 M NaCl followed by 2 L distilled H_2O and stored at 4°C until required.

2.2.2. Aluminium Hydroxide Gel

The method was adapted from that of Ikemori et al, (1975). 50 ml 0.2 M aluminium sulphate were heated to 63°C. 5 ml 58% ammonium hydroxide were added to 45 ml distilled H_2O previously heated to 67°C. The ammonium hydroxide was then poured into the aluminium sulphate in a steady stream. The temperature was maintained at 62-63°C for 10 min while the mixture was stirred. The gel was then washed with distilled H_2O until no free sulphate could be detected by testing with 0.1 M barium nitrate.

2.2.3. Purification of Kabi Fibrinogen

Kabi fibrinogen was dissolved in distilled H_2O to a concentration of 20 mg/ml and stored in aliquots at -20°C until required. Before use the fibrinogen was diluted to 5 mg/ml with 0.05 M Tris/HCl buffer pH 8.6 made 0.05 M with respect to NaCl and 2.67 mM with respect to $CaCl_2$. Lysine Sepharose gel (4 g wet weight to every 100 mg fibrinogen) was added to this solution and stirred gently for 4 h at 4°C to remove plasminogen. After filtration the solution was dialysed overnight at 4°C against the same Tris/NaCl/$CaCl_2$ buffer. At 4°C there was an extensive precipitate which almost completely redissolved on warming at 37°C for 5 min. Any remaining precipitate was removed by centrifugation and discarded. The supernatant was applied to a column (1.5 x 30 cm) of DEAE-cellulose equilibrated with the same Tris/NaCl/$CaCl_2$ buffer.

Initially elution was carried out using a gradient of 0.05 -0.5 M NaCl in the Tris/NaCl/$CaCl_2$ buffer. However the same elution profile was obtained using the equilibration buffer followed by the same buffer made 0.1 M with respect to NaCl and then 1.0 M with respect to NaCl. 3 ml fractions were collected and the column eluted at a flow rate of 36 ml/h. The absorption at 280 nm of each fraction was measured and aliquots from each peak examined on SDS-PAGE and tested for the presence of plasminogen and factor XIII.

2.2.4. Purification of Fibrinogen from Plasma

An outline of the procedure is shown in Figure 5. A normal preparation used 100 ml plasma which, if necessary, was centrifuged before use to remove insoluble lipid components. The plasma was applied to a lysine-Sepharose column (1 x 20 cm) equilibrated with 0.05 M Tris/HCl buffer pH 7.5. Effluent from the column was collected until the absorbance at 280 nm was less than 0.1. Aluminium hydroxide gel was added, 1 part to 10, to the plasminogen-free plasma. After mixing, the solution was allowed to stand for 15 min at 37^{o}C before the gel was removed. by centrifugation at 1000 g for 15 min on an MSE 6L centrifuge. The volume of plasma was then adjusted to twice the starting plasma volume and a 25% ammonium sulphate fractionation made. After 10 min at room temperature the precipitate was removed by centrifugation at 1000 g and washed with a 23% saturated ammonium sulphate solution. This suspension was centrifuged at 1000 g and the precipitate resuspended in 0.05 M Tris buffer pH 8.6 made 0.05 M with respect to NaCl and dialysed overnight against the same buffer. The protein solution was applied to a DEAE-Cellulose column (1.5 x 30 cm) equilibrated with the same Tris/NaCl buffer and eluted first with the same Tris/NaCl buffer followed by the same buffer made 0.1 M with respect to NaCl and finally the same buffer made 1.0 M with respect to NaCl. 6 ml fractions were collected and the column eluted at a flow rate of 36 ml/h. The absorption at 280 nm of each fraction was measured and aliquots from each peak were examined by SDS-PAGE and tested for the presence of plasminogen and factor XIII.

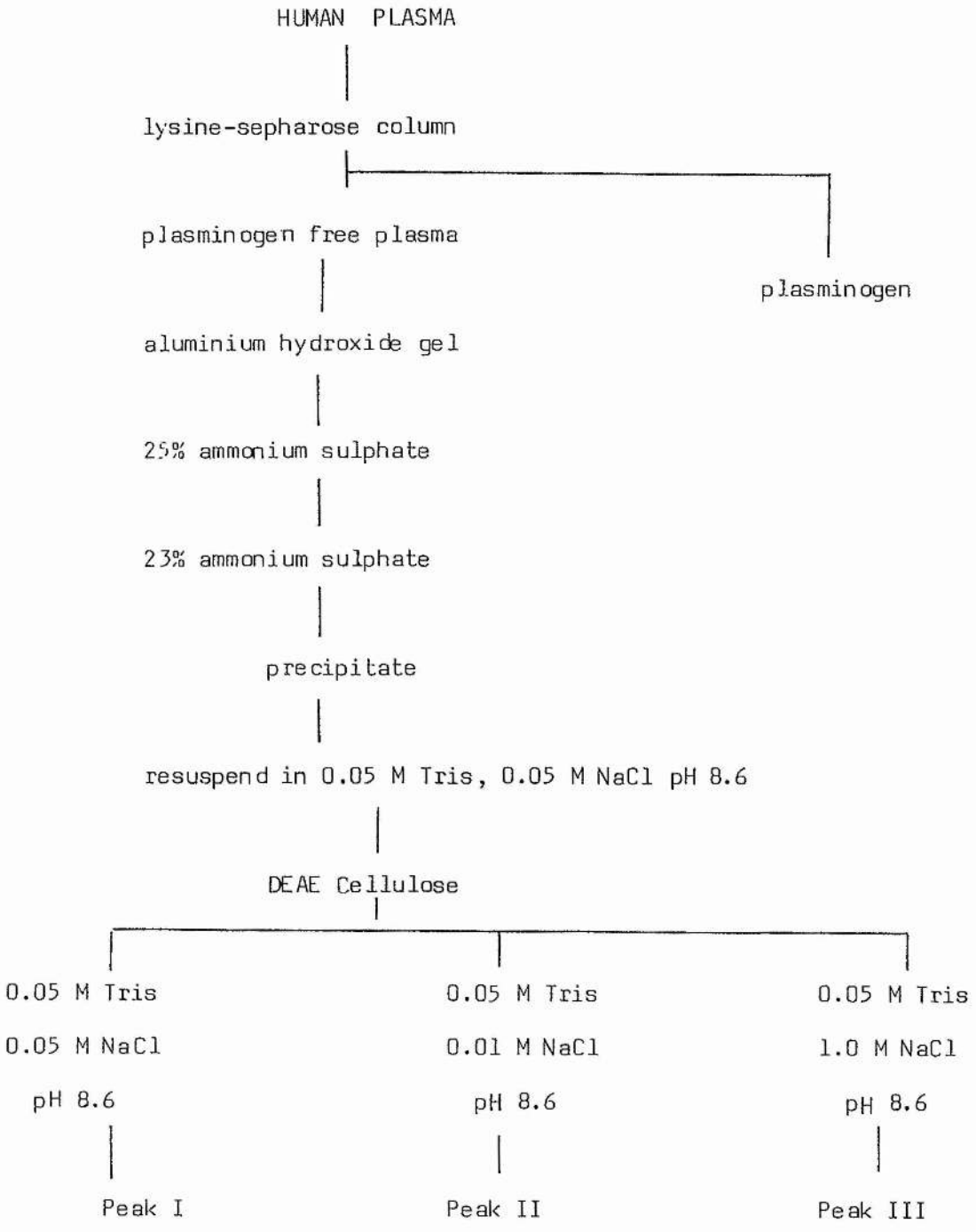


Figure 5: Purification Procedure to obtain Fibrinogen

The supernatants following precipitation with 23% saturated and 25% saturated ammonium sulphate were dialysed overnight against 0.05 M Tris buffer pH 7.5 at 4°C. The solutions were then examined by immunoelectrophoresis to determine whether appreciable quantities of fibrinogen were being lost at these stages.

To protect against degradation during purification procedures and long dialyses a number of inhibitors were added to the solutions to be dialysed. These were -

INHIBITOR	FINAL CONCENTRATION	REFERENCE
Benzamidine	8 mM	F. Markwardt et al. (1968)
N-Ethyl maleimide	10 mM	J.D. Gregory (1955)
Phenylmethyl sulphonylfluoride	1 mM	D.E. Fahrney & A.M. Gold (1963)

A stock solution of 50 mM phenylmethylsulphonylfluoride in isopropanol was prepared and stored at 4°C. N-ethyl maleimide and benzamidine were added as solids to the solution to be dialysed.

2.2.5. Purification of Plasminogen from Plasma

Plasminogen was purified from fresh frozen human plasma by a modification of the method of Deutsch & Mertz (1970). A lysine-Sepharose column (1 x 20 cm) was equilibrated with 0.05 M Tris/HCl buffer pH 7.5. 100 ml plasma was passed through the column at a flow rate of 40 ml/h. The column was then washed with 0.05 M

Tris/HCl pH 7.5 made 0.5 M with respect to NaCl before the plasminogen was eluted using 0.2 M 6-amino-n-hexanoic acid. The column was regenerated by washing first with 0.05 M Tris/HCl pH 7.5 made 0.2 M with respect to 6-amino-n-hexanoic acid and 1.0 M with respect to NaCl followed by 0.05 M Tris/HCl pH 7.5.

Plasminogen was freed from 6-amino-n-hexanoic acid by passing it through a G-25 Sephadex column equilibrated with 0.05 M Tris/HCl pH 7.5. The resulting plasminogen solution was stored in small aliquots at -20^{o}C after determination of the plasmin activity according to the caseinolytic method of Johnson et al. (1969).

2.3. ANALYTICAL METHODS

2.3.1. Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was carried out essentially according to the methods of Weber & Osborn (1969) and Weber et al. (1972). Sodium dodecyl sulphate polyacrylamide gel electrophoresis was used to -

a) determine the intactness of fibrinogen

b) estimate molecular weights

c) examine the degree of cross-linking of fibrinogen

2.3.1.1. Acrylamide Solutions

A number of acrylamide solutions, giving different degrees of cross-linking and hence separating different ranges of molecular weights, were used. For each gel type the appropriate quantities

of acrylamide and bisacrylamide, shown in Table I, were dissolved in 200 ml gel buffer. The solution was stored at $4^{o}C$ until required.

% Acrylamide	Acrylamide (grams)	Bisacrylamide (grams)
10	19.4	0.6
5	9.5	0.5
4	7.68	0.32
3	5.82	0.18

Table I Quantities of acrylamide and bisacrylamide required for 200 ml of Acrylamide solution.

2.3.1.2. Gel Buffer

6 M urea

0.2% SDS

0.1 M Tris/HCl pH 7.4

2.3.1.3. Preparation of Gels

19 ml gel (acrylamide/bisacrylamide) solution were mixed with 1 ml freshly prepared ammonium persulphate solution (150 mg/10 ml distilled H_2O) and 30 µl TEMED. Glass gel tubes of length 11 cm

and internal diameter of 4 mm were used. The gel solution was added to the tubes and overlaid with distilled H_2O until gelled. The H_2O was replaced with gel buffer and the gels left overnight at 4^oC before use.

2.3.1.4. Chamber Buffer

Chamber buffer was prepared as a concentrated stock solution and before electrophoresis was diluted 1 : 10 with distilled H_2O.

2% SDS

1.0 M Tris/HCl pH 7.4

2.3.1.5. Running Conditions

For reduced samples, samples were boiled for 5 min with an equal volume of reducing agent: 8 M urea

3% SDS

3% mercaptoethanol

For non-reduced samples an equal volume of 3% SDS/8 M urea solution was added and the samples left at room temperature for 10 min. To each sample, 10 µl of 1 mg/ml protein or equivalent, was added one drop of Bromophenol Blue (0.05%(w/v) in distilled H_2O) as a tracking dye and one drop of glycerol. Samples were applied to gels after removing the gel buffer overlay. Electrophoresis was carried out at 5-8 mA per tube and $100V_{max}$ until the marker dye was about 1 cm from the bottom of the tube.

2.3.1.6. Standard Protein Solutions

10 µl of 1 mg/ml solutions of at least three of the proteins shown below in Table II were electrophoresed under the same conditions as samples whose molecular weights were to be determined.

Protein	Molecular Weight
Phosphorylase b	92,500
Bovine Serum Albumin	66,000
Myoglobin	17,200
Cytochrome C	12,500

Table II Molecular weights of Standard Proteins used in SDS-PAGE for molecular weight determination

2.3.1.7. Protein Stain

3.5 g Brilliant Blue R

900 ml ethanol

900 ml distilled H_2O

200 ml glacial acetic acid

2.3.1.8. Destaining Solution

1350 ml distilled H_2O

500 ml ethanol

150 ml glacial acetic acid

2.3.1.9. Molecular Weight Determination

Destained gels were scanned on a Vitatron TLD 100 densitometer using a 570 nm filter for gels stained for protein. The mobility of each band was calculated using the formula:

$$\text{mobility} = \frac{\text{length of gel before staining}}{\text{distance moved by marker dye}} \times \frac{\text{distance moved by protein}}{\text{length of gel after staining}}$$

The mobilities of standard proteins were plotted against the $\log_{10}$ of their molecular weight and the molecular weight of the unknowns obtained by reading off from the calculated mobilities.

2.3.2. Test for the Presence of Plasminogen

Streptokinase was added to a sample of fibrinogen to a final concentration of 2000 IU (International Units) per ml and the sample incubated at 37°C overnight. Samples were examined for proteolytic degradation by SDS-polyacrylamide gel electrophoresis.

2.3.3. Test for the Presence of Factor XIII

Test samples were incubated with a final concentration of 5 N.I.H. (National Institute of Health) units for thrombin per ml and 20 mM $CaCl_2$ for two hours at 37°C. The presence of cross-linked γ- chains after SDS-polyacrylamide gel electrophoresis of the reduced sample indicated the presence of factor XIII.

2.3.4. Determination of Fibrinogen Concentration

The concentration of fibrinogen solutions was calculated from absorbance readings at 280 nm on a Cecil CE 272 UV Spectrophotometer. The $E_{1\%}$ value for fibrinogen at 280 nm was taken as 15.1 (Marder et al., 1969).

2.3.5. Immunoelectrophoresis

1% agarose in Tris/phosphate buffer (0.039 M Tris, 0.05 M H_3PO_4, 0.05 M NaCl) pH 8.6 was autoclaved at 15 psi for 20 min. Three glass microscope slides were flooded with 12 ml molten agarose. When set, troughs and wells were cut in the agar surface and the wells removed. 1-2 µL of sample were added to the wells and electrophoresed for 4 h at 150 V. The electrophoresis buffer

was the same Tris/phosphate buffer in which the agar was dissolved. After electrophoresis the agar troughs were removed and 10 uL antiserum, either sheep antisera to whole human serum or sheep antiserum to human fibrinogen, were added. The slides were then left for 24 h at room temperature in a damp chamber to allow diffusion of antiserum. The slides were then washed for 48 h with one change of 1% NaCl followed by washing in distilled H_2O for 24 h. They were then dried overnight in an oven at $37^{o}C$ before being stained with Brilliant Blue(2.3.1.7.) for 5 min and destained in ethanol/acetic acid/ H_2O(2.3.1.8.).

2.3.6. Cross-Linking of Fibrinogen

2.3.6.1. Cross-Linking Reagent

Cross-linking was carried out using dimethyl suberimidate dihydrochloride (DMS). Prior to use DMS was dissolved in 0.5 M triethanolamine hydrochloride (TEA) adjusted to pH 8.5 with 8 M NaOH (Furlan et al., 1977).

2.3.6.2 Effect of DMS Concentration

Initial experiments were performed to find the optimun DMS concentration which gave maximum intra-molecular cross-linking with minimum inter-molecular cross-linking at a fibrinogen concentration of 1 mg/ml. A concentrated stock solution of 12 mg/ml DMS was prepared and the appropriate volume added to 0.2 ml of 5 mg/ml fibrinogen in 0.05 M Tris/HCl pH 8.6 made 0.05 M with respect to NaCl. The solution

was adjusted to a final volume of 1 ml with 0.5 M TEA pH 8.5 and incubated for 1 h at 30°C. Before running on SDS-PAGs the solutions were dialysed either for 4 h at room temperature or overnight at 4°C to remove the products of hydrolysis of DMS.

2.3.6.3. Effect of Calcium/EGTA

When the effect of calcium on cross-linking was investigated 0.4 ml of 2.5 mg/ml fibrinogen in 0.05 M Tris/HCl buffer pH 8.6 made 0.05 M with respect to NaCl was mixed with either -

a) 0.5 ml of 0.05 M Tris/HCl pH 8.6 made 0.05 M with respect to NaCl for cross-linked fibrinogen control

b) the above buffer made 4 mM with respect to $CaCl_2$ for cross-linked fibrinogen+Ca

c) the above buffer made 4 mM with respect to EGTA for cross-linked fibrinogen+EGTA

and incubated for 1 h at 30°C before the addition of 0.1 ml of 5 mg/ml DMS. Products of cross-linking were examined by SDS-PAGE.

2.3.6.4. Extraction of Protein from SDS-PAGs

The method was that of Bray and Brownlee (1973). SDS-PAGs were stained with Brilliant Blue and after destaining the protein-containing bands were cut-out with a razor blade. Bands of the same mobility from 10-15 gels were chopped into small fragments and pooled. The protein was extracted into 5 ml chamber buffer (2.3.1.4.) by gentle shaking at 37°C overnight. If necessary a second extraction was performed.

Gel fragments were removed by filtration and the protein precipitated with KCl at a final concentration of 0.2 M. After 10 min at $0^{o}C$ the precipitate was removed by spinning on a bench centrifuge for 20-30 min. The precipitate was washed with acetone containing 0.1 M HCl and once with acetone alone to remove the protein dye. The precipitate was then dried.

2.3.6.5. N - Terminal Analysis

The method used was adapted from Woods & Wang (1967). 5-10 nmole protein were taken up in 50 mM $NaHCO_3$ pH 7.5. 30 ml dansyl chloride (2.5 mg/ml in acetone) were added and the reaction mixture left for 30 min on a heating block at $50^{o}C$. The sample was then dried by passing N_2 over it. 100 µl 6N HCl were added to the dried sample and the tube sealed. Hydrolysis was carried out at $105^{o}C$ overnight. The hydrolysis tubes were opened and the samples dried. The solid residue was extracted twice with the upper phase of ethylacetate/H_2O (1:1). The extract was dried and redissolved in 50% aqueous pyridine and examined by 2-dimensional thin layer chromotography on Polyamide sheets using 1.5% formic acid as solvent in the first dimension and benzene/glacial acetic acid (9:1) in the second dimension.

2.3.7. Electrophoresis of ^{45}Ca-Fibrinogen

1 µCi $^{45}CaCl_2$ was added to 0.5 ml 1 mg/ml fibrinogen in

0.05 M Tris buffer pH 7.5 made 0.5 M with respect to NaCl and the solution left for 4 h at 4°C. Excess calcium was removed by passing through a small G-25 Sephadex column (Neal & Florini, 1973). Fibrinogen was reduced by adding an equal volume of reducing agent (2.3.1.5.) and leaving at 15°C for 1 h. Electrophoresis was carried out as in (2.3.1.) using 5% acrylamide gels.

Some gels were stained in the normal manner for protein others were cut into 0.5 cm slices. Each slice was added to 0.5 ml H_2O_2 and left at 60°C overnight to dissolve the gel. 5 ml Packard Pico Fluor ^{TM}15 scintillation fluid was added to each sample and counted for 20 min in an Inter-technique Scintillation Spectrometer Model SL 30.

2.3.8. Plasmin Digestion of ^{45}Ca-Fibrinogen

Fibrinogen, purified from Kabi fibrinogen, was dialysed at 4°C against 0.05 M Tris/HCl buffer pH 7.5 made 0.05 M with respect to NaCl and 5 mM with respect to EDTA to remove calcium. EDTA was removed by dialysing at 4°C against the same buffer without EDTA.

In a typical experiment 1 ml 1 mg/ml 'calcium-free' fibrinogen was diluted with an equal volume of the Tris/NaCl buffer made 10^{-2}M with respect to $MgCl_2$ and 10^{-4} M with respect to $CaCl_2$ and 10 µl 0.2 mM ^{45}CaCl were added. The solution was left at 4°C for 4 h before digestion by plasmin.

Fibrinogen was digested by adding 1.05 CTA (Committee on Thrombolytic Agents) units of plasmin and 175 I.U. (International Units) streptokinase for every 5 mg fibrinogen. Plasminogen was activated to plasmin by adding the streptokinase to plasminogen in 25% glycerol in 0.05 M Tris/HCl pH 7.5 buffer and placing the solution at 37°C for 15 min before use. The plasmin digestion of fibrinogen was stopped by adding 250 units of Trasylol per unit of plasmin.

Excess calcium and small peptides were removed by passing through a G-25 Sephadex column (Neal & Florini, 1973). Samples of filtered fibrinogen and plasmin digested fibrinogen were counted on a Intertechnique Liquid Scintillation Spectrometer Model SL-30. The scintillation fluid used was Toluene based scintillator containing (4 g/L PPO, 0.5 g/L POPOP)/Triton X-100 2:1. 100 ul aqueous sample were added to 10 ml scintillation fluid.

2.3.9. Flow Dialysis

2.3.9.1. The Flow Dialysis Cell

The flow dialysis cell, similar to that described by Colowick & Womack (1969), was obtained from Bel Art Products Ltd. The lower chamber had a volume of 2.8 ml and was completely filled with buffer. Flow through the lower compartment was regulated

by means of an adjustable hydrostatic head and a flow rate of 8 ml/min was normally employed. The upper compartment, to which the protein and ligands were added, was enlarged to give a volume of 1.5 ml. The two compartments were separated by a square of dialysis membrane. In later experiments the cell was modified by clamping the dialysis membrane between two pieces of perspex which had nine 3 mm diameter holes drilled in the central area which reduced the area of membrane exposed to solvent. All experiments were performed at room temperature.

2.3.9.2. Dialysis Membranes

Three types of dialysis membrane were tested; Visking tubing (32/32 from Scientific Instrument Centre Ltd), dialysis sacks (35 mm from Sigma) and dialysis sheets (Bel Art Products Ltd). Visking tubing and Sigma tubing were extensively studied as both single and double layers which had been treated in one of the following ways:

a) washed in dialysis buffer for 4 h before use

b) boiled for 30 min in 1% sodium bicarbonate followed by washing with distilled water and treatment with 10^{-3} M EGTA solution for 15 min. The tubing was then extensively washed and soaked in dialysis buffer before use.

c) acetylated by the method of Craig & Konigsberg (1961). A length of dialysis tubing was wetted with distilled water and knotted at one end. The

dialysis sack was filled with pyridine/acetic anhydride (9:1) solution and held upright inside a cylinder, filled with the same pyridine/acetic anhydride solution, so that both sides of the tubing were in contact with the acetylating solution. After 16 h at room temperature the tubing was washed with 1% acetic acid to remove pyridine. Washing was complete when two subsequent washes gave the same absorbance reading at 260 nm. Tubing was stored at $4^{o}C$ in 50% ethanol and was soaked in dialysis buffer before use.

To reduce the loss of calcium from the upper chamber the flow cell was modified by using an insert which reduced the membrane surface area exposed to the solvent by 75%. This system was tested in the same manner as above using a single layer acetylated Sigma membrane 0.05 M Tris/HCl buffer pH 7.5 made 0.15 M with respect to NaCl and 10 mM with respect to $MgCl_2$ was used and 2 μCi $^{45}CaCl_2$ added in a volume of 5 μl.

The modified flow cell was used in ligand binding experiments and reverse ligand binding experiments with single layer acetylated Sigma membrane.

2.3.9.3. Preliminary Experiments

A single or double layer of one of the dialysis membranes was

used. 1 ml 0.05 M Tris/HCl buffer pH 7.5 made 0.15 M with respect to NaCl and made 10^{-2} M with respect to $MgCl_2$ was added to the top compartment and the same buffer flowed through the bottom compartment to a Uniscil-UFC 120 fraction collector. 1 µCi $^{45}CaCl_2$ in 10 uL of solution was added to the top chamber and in some experiments subsequent additions of $^{40}CaCl_2$ (10 µL of 1 mM $CaCl_2$) were made after 10-15 fractions had been collected. Fractions were collected every 30 seconds and the radioactivity in the effluent measured by adding 1 ml from each fraction to 10 ml Packard Pico Fluor ^{TM}15 scintillation fluid.

All samples were monitored by liquid scintillation counting in an Intertechnique SL-30 Spectrometer.

2.3.9.4. Plasmin Digestion of Fibrinogen

A single layer of acetylated Sigma membrane separated the two compartments in the flow dialysis cell and 0.05 M Tris/HCl buffer pH 7.5 made 0.15 M with respect to NaCl and 10^{-2}M with respect to $MgCl_2$ flowed through the lower chamber. 1 ml of 5 mg/ml fibrinogen was added to the top chamber and to this was added 1 µCi $^{45}CaCl_2$ in a volume of 10 µL. After 3 min 20 µL plasmin solution (50 µL plasminogen (0.75 CTA u/ml), 20 µL streptokinase (2000 u/ml) and 3 drops glycerol placed in a 37°C water bath for 15 min) was added. Fractions were collected every 30 sec and 0.5 ml from each fraction was added to 5 ml Packard Pico Fluor ^{TM}30 scintillation fluid. Small

aliquots (5 µL) were removed from the top chamber at intervals and the extent of digestion examined by SDS-PAGE.

A buffer control in which $^{45}CaCl_2$ was added to 1 ml buffer in the upper chamber and a protein control in which the plasminogen was replaced by buffer were also performed.

2.3.9.5. Preparation of Calcium-free Solutions

Ligand binding experiments required 'calcium-free' fibrinogen. Fibrinogen which had been prepared from plasma (2.2.4) was lyophylised and redissolved in the buffer to be used for flow dialysis. This was one of 0.05 M Tris/HCl pH 7.5 made 0.15 M with respect to NaCl the same Tris/HCl/NaCl buffer made 10 mM with respect to $MgCl_2$ or 0.05 M imidazole/HCl pH 7.5 made 0.15 M with respect to NaCl. The protein solution was then dialysed first against the appropriate buffer made 1 mM with respect to EGTA then against two changes of the same buffer without EGTA. The proteinase inhibitor solution (2.2.4.) was added to the fibrinogen solution before dialysis against EGTA buffer.

The calcium concentrations of protein and buffer solutions were determined by Atomic Absorption Spectrometry (AAS) on a Techtron AA4 Atomic Absorption Spectrometer. When determining calcium concentrations in protein solutions the method of standard additions was used.

As the buffers were found to be contaminated with calcium, solutions of the individual components of the buffers, ten times more concentrated than the final buffer concentration, were passed through

a 20 X 2 cm Chelex-100 column. $MgCl_2$ solutions could not be treated in this manner because of the properties of the Chelex-100 gel. Dialysis sacks were pretreated in different ways (2.3.9.2.) and experiments were performed to determine if this had any influence on the final calcium concentrations of the protein solutions.

2.3.9.6. Ligand Binding

The flow cell was as described previously and the buffer systems mentioned above were used. 1 ml fibrinogen with a concentration of 5 to 9 mg/ml was added to the top chamber followed by 8 µCi $^{45}CaCl$ (5-10 µL). Once a steady state was attained a small volume of unlabelled ligand was added (5-10 µL of 1 mM $CaCl_2$). Similar additions were made 10 to 12 times and after each addition a new steady-state level of isotope in the effluent was reached which reflected the free calcium concentration in the upper chamber. The final ligand addition was a large excess (10 µL 2 M $CaCl_2$) relative to the protein concentration and this steady-state value corresponded to that obtained with labelled substrate alone.

To evaluate the binding constant the data obtained from the above was treated as follows: the steady state value of isotope in the effluent for any given ligand concentration was taken as a measure of the free ligand concentration in the upper chamber. The plateau value obtained with excess unlabelled ligand was that corresponding to 100% of the ligand in the free state. From this free(F) and bound(B)

calcium concentrations were derived.

The dissociation constant and the number of calcium binding sites can be evaluated by plotting B/F against B according to Scatchard (1948). According to the Scatchard equation

$$\frac{B}{F} = K_a(n - B) \qquad .. \quad .. \quad .. \quad .. \quad (1)$$

Where B = concentration of bound ligand

F = concentration of free ligand

K_a = association constant of the ligand/protein complex

n = number of ligand binding sites

In order that the number of ligand molecules bound can be read directly from the Scatchard plot the bound values are divided by the protein concentration giving

B = mol ligand bound per mol protein

Equation (1) can be re-written in the linear form, $y = mx + c$ as

$$\frac{B}{F} = -K_a B + nK_a$$

and a plot of $\frac{B}{F}$ against B will give a straight line providing K is constant. The gradient K_a corresponds to the reciprocal value of the dissociation constant K_d and the intercept of the x - axis gives the value for n.

2.3.9.7 Reverse Ligand Binding

An alternative way of measuring ligand binding constants is by reverse ligand binding where the ligand is removed from the system by a chelating agent such as EGTA. The method is similar to that for ligand binding and was first described by Haeich et al (1980).

1 ml of fibrinogen, of known protein and calcium concentrations, was added to the top chamber of the flow dialysis cell. To this was added 8 μCi $^{45}CaCl_2$. 10-12 additions of EGTA (5-10 μL of 1 mM solution) were made and each time a new steady-state level of isotope in the effluent was established. This was followed by a 20-fold excess of EGTA which in turn was followed by a 20-fold excess of calcium ions over EGTA. From this a plot of CPM against fraction number could be made. This data was used to determine the free and protein bound concentrations of the ligand, as described by Haeich et al.and the resulting data analysed according to Scatchard.

The additions of excess calcium ions and EGTA are made so that the diffusion rates of calcium ions and the EGTA-calcium ion complex can be evaluated. The concentrations of labelled isotope in the effluent at different EGTA concentrations are used to calculate bound and free calcium ion concentrations as follows -

let f = flow rate through lower chamber

D_1 = diffusion rate of ligand (L)

D_2 = diffusion rate of the chelator-ligand complex (EL)

L = free ligand concentration in the upper chamber

L_T = total (bound+free) ligand concentration in the upper chamber

L_T^* = the radioactive ligand concentration in the upper chamber

C^* = radioactive ligand concentrations in the collected fractions

then

$$C^* = \frac{D_1}{f} \cdot \frac{L_T^*}{L_T}(L) + \frac{D_2}{f} \cdot \frac{L_T^*}{L_T}(EL) \quad \ldots\ldots\ldots (2)$$

The association constant of the chelator

$$K = \frac{(EL)}{(E)(L)}$$

and

$$E_T = (E) + (EL)$$

by substitution into (2)

$$C^* = \frac{D_1}{f} \cdot \frac{L_T^*}{L_T}(L) + \frac{D_2}{f} \cdot \frac{L_T^*}{L_T} \cdot \frac{E_T}{1 + \frac{1}{K(L)}} \quad \ldots\ldots (3)$$

when excess chelator is added, $E_T >> L_T$, then L is very small

and

$$(EL) \simeq L_T$$

therefore

$$C^* \simeq \frac{D_2}{f} \cdot L_T^*$$

i.e.

$$D_2 \simeq \frac{C^* f}{L^*_T}$$

when excess ligand is added, $L_T \gg E_T$, then $L \simeq L_T$

and

$$C^* \simeq \frac{D_1}{f} \cdot L^*_T$$

i.e.

$$D_1 \simeq \frac{C^* f}{L^*_T}$$

Thus D_1 and D_2 can be evaluated for any particular series of results. The association constant for the calcium-EGTA complex was taken as $10^{11}M^{-1}$ as calculated by Owen (1976) who determined that this value was independent of the buffer system used.

Equation (3) can now be re-written in terms of constants and variables so that it can be solved for (L) the free ligand concentration in the upper chamber.

Let

$$C^* = V_1$$

$$E_T = V_2$$

$$\frac{D_1}{f} \cdot \frac{L^*_T}{L_T} = C_1$$

$$\frac{D_2}{f} \cdot \frac{L^*_T}{L_T} = C_2$$

$$\frac{1}{K} = C_3$$

equation (3) then becomes

$$V_1 = C_1(L) + \frac{C_2 V_2}{\frac{1+C_3}{(L)}}$$

which can be re-arranged into the quadratic form, $ax^2 + bx + C = 0$

as

$$C_1(L)^2 + (C_1C_3 + C_2V_2 - V_1)(L) - V_1C_3 = 0$$

then

$$(L) = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a} \qquad .. \quad .. \quad .. \quad (4)$$

where

$$a = C_1$$

$$b = C_1C_3 + C_2V_2 - V_1 \qquad .. \quad .. \quad .. \quad (5)$$

$$c = (-V_1C_3)$$

therefore, by inserting the value of C^* and E_T for each plateau value a series of (L) values is obtained.

To calculate the value of n, the moles of ligand bound per mole of protein, the equation -

$$L_T = (L) + (EL) + M_T n$$

is used.

where

M_T = protein concentration in the upper chamber

therefore

$$n = \left[L_T - (L) - \frac{(E_T)}{1 + \frac{1}{K(L)}} \right] . \; M_T^{-1}$$

Once n has been calculated for the series of (L) values a Scatchard plot of bound/free against bound can be made

where

(L) = free ligand concentration

n = molar ratio of bound ligand to protein.

RESULTS

3.1. PURIFICATION OF KABI FIBRINOGEN

It has been suggested that the carboxyl terminal of the Aα - chain of fibrinogen may be involved in calcium binding and it is known that this part of the fibrinogen molecule is susceptible to degradation by plasmin in vivo. It was important, therefore, to prepare as intact a fibrinogen preparation as possible for use in calcium binding studies.

Human fibrinogen (Grade L) from Kabi Pharmaceuticals was found, when examined by SDS-PAGE to have a heterogeneous Aα - chain and a high molecular weight contaminant thought to be fibronectin. Traces of Factor XIII and plasminogen also appeared to be present.

As it was desirable to work at physiological calcium concentrations purification schemes utilising phosphate buffers (Mosesson & Finlayson, 1963) could not be used, therefore, purification using Tris/HCl buffers was investigated.

The elution profile of Kabi fibrinogen from DEAE-Cellulose is shown in Figure 6a and the electrophoretic pattern of reduced samples from each of the three peaks is shown in Figure 6b. Each peak was tested for the presence of plasminogen and Factor XIII and results are shown in Figure7a and Figure 7b respectively.

Peak I was fibrinogen with very little degradation apparent. This peak had no detectable plasminogen or Factor XIII. Peak II was a fibrinogen with an appreciable amount of Aα - chain degradation which was detectable as a heavy Bβ - band when compared to the

Figure 6: Purification of Kabi Fibrinogen on DEAE-Cellulose

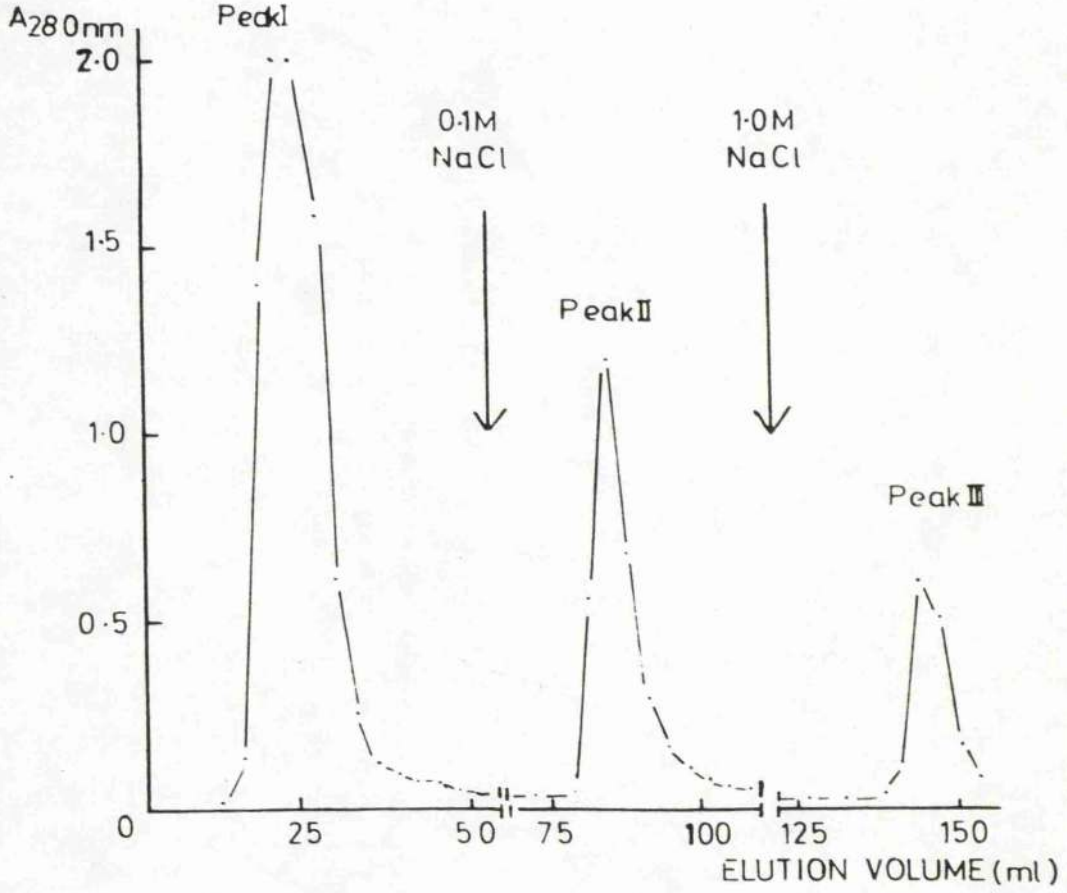
a) elution profile of Kabi Fibrinogen from DEAE-Cellulose

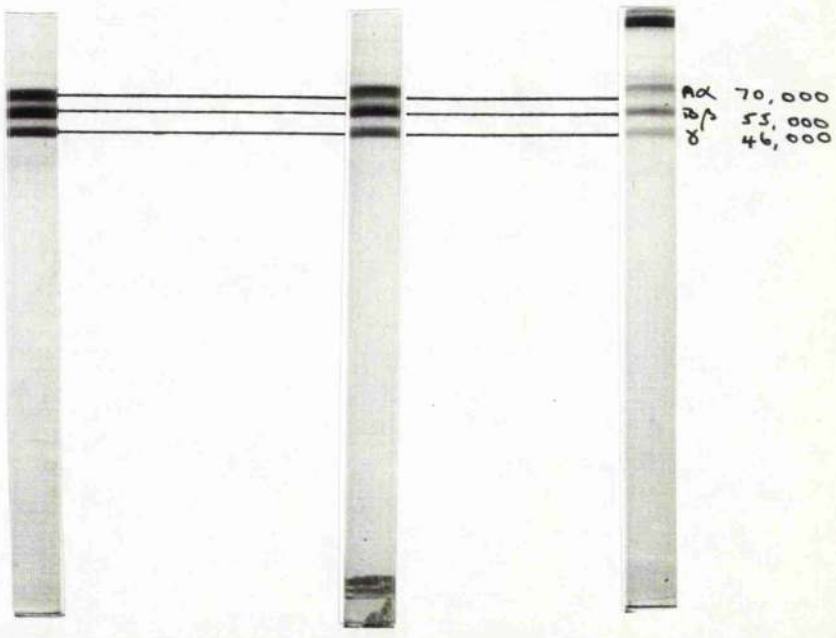
b) 10% SDS-PAGs of reduced samples from -

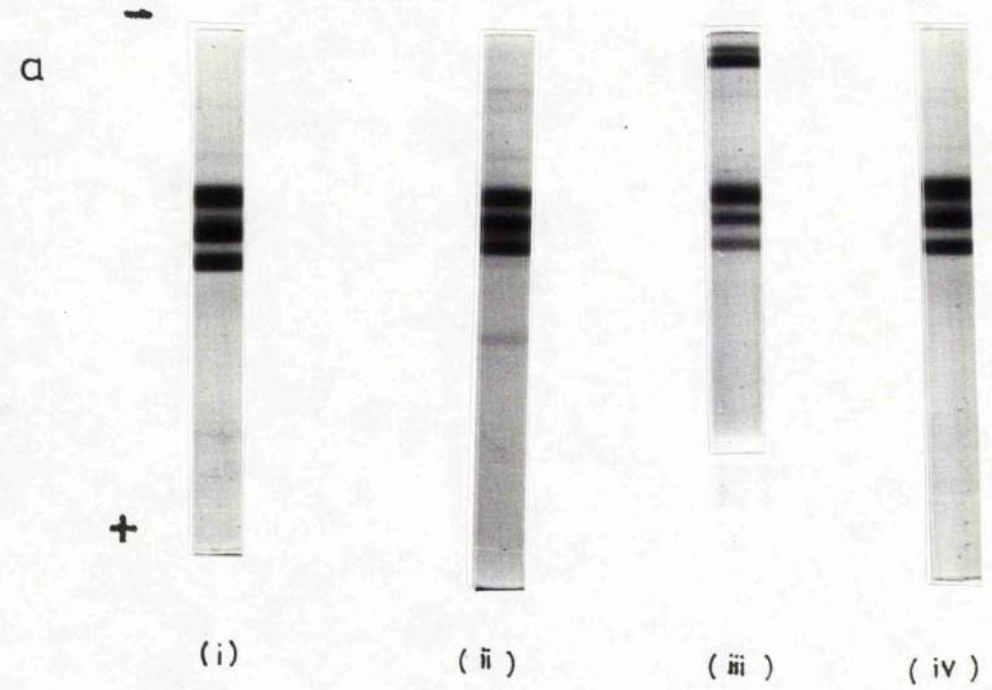
i) Peak I

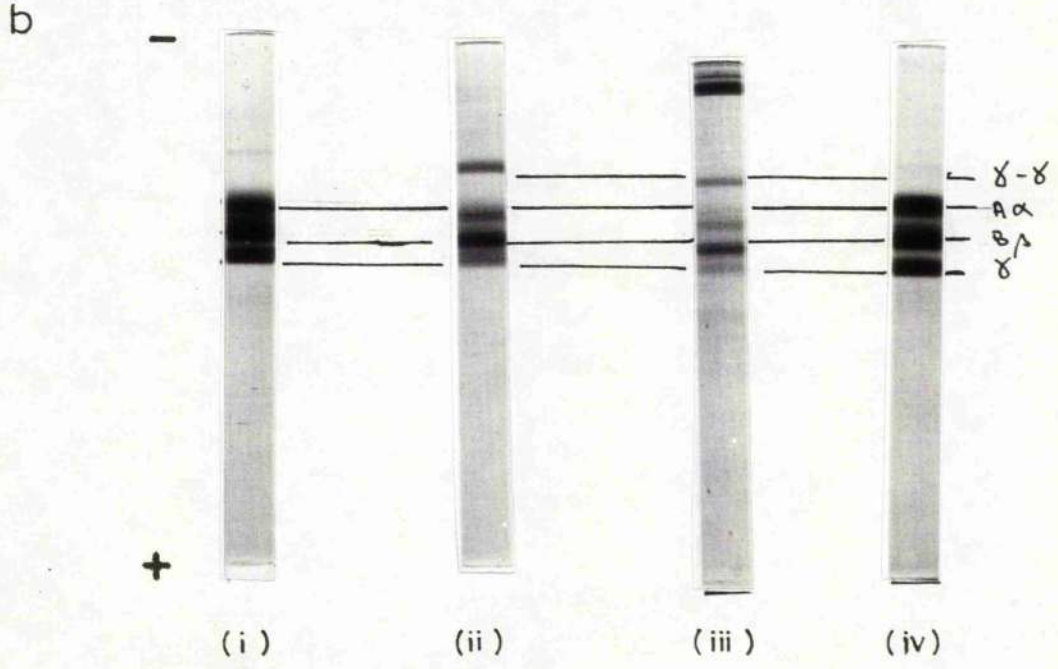
ii) Peak II

iii) Peak III


A280nm
2·0
1·5
1·0
0·5
0
Peak I
0·1M NaCl
1·0M NaCl
Peak II
Peak III
25
50
75
100
125
150
ELUTION VOLUME (ml)


α 70,000
β 55,000
γ 46,000


a
-
+
(i)
(ii)
(iii)
(iv)


b
-
+
γ-γ
Aα
Gγ
(i)
(ii)
(iii)
(iv)

γ- bands. There were significant amounts of Factor XIII present as determined by the presence of $\gamma - \gamma$ dimers after digestion of fibrinogen with thrombin (Figure 7b). Peak III had a high proportion of a high molecular weight component with lower mobility than fibrinogen on SDS-PAGs. After reduction the mobility increased but there was still one band. This material could be fibronectin which is known to associate with fibrinogen (Yamada & Olden, 1978). No plasminogen was detected in any of the three peaks (Figure 7a) as would be expected after initial treatment of the fibrinogen solution with lysine-sepharose.

Recovery of protein from the column was 68.7 $\pm$ 1.6%. Percentage yield in each peak is shown in Table III.

Peak	% Yield $\pm$ A.D.
I	69.1 $\pm$ 4.0
II	19.2 $\pm$ 2.8
III	11.7 $\pm$ 1.6

Table III: Percentage yield of Protein from DEAE-Cellulose. Chromatography of Kabi Fibrinogen.

The quality of Kabi fibrinogen was found to be variable and in those batches purchased latterly the recovery from purification decreased to approx 50% of the applied protein although the proportional yield of peak I fibrinogen remained the same. It was

Figure 7: Tests on Fractionated Kabi Fibrinogen

a) plasminogen test: 5% SDS-PAGs of reduced samples of

i) Peak I
ii) Peak II
iii) Peak III
} from DEAE-Cellulose chromatography

iv) standard fibrinogen

b) Factor XIII test: 5% SDS-PAGs of reduced samples of

i) Peak I
ii) Peak II
iii) Peak III
} from DEAE-Cellulose chromatography

iv) standard fibrinogen

decided therefore to modify the purification procedure used for Kabi fibrinogen and apply it to dated blood-bank fresh frozen plasma obtained from The Blood Transfusion Service, Ninewells Hospital, Dundee.

3.2. PURIFICATION OF FIBRINOGEN FROM PLASMA

Figure 8 shows the elution profile of fibrinogen, obtained from human plasma, purified on DEAE-Cellulose and SDS-PAGs of a reduced sample from each of the three peaks eluted from the column. The results of the tests for plasminogen and Factor XIII are shown in Figures 9a and 9b respectively. Peak I fibrinogen had little sign of degradation and was apparently free from Factor XIII. Peak II fibrinogen showed signs of Aα-chain degradation and was contaminated with Factor XIII. Peak III consisted mainly of two contaminants one comparable with the high molecular weight contaminant found in Kabi fibrinogen, thought to be fibronectin, and the other, with molecular weight 67,000 possibly albumin. Plasminogen was not detected in any of the three peaks as would be expected after pre-treatment of the plasma with lysine-sepharose. The recovery from the DEAE-Cellulose column was 61.1$\pm$ 2.4% and the percentage protein in each peak is shown in Table IV.

Figure 8: Purification of Fibrinogen from Human Plasma

The elution profile of human fibrinogen from DEAE-Cellulose and 10% SDS-PAGs of a reduced sample from each peak

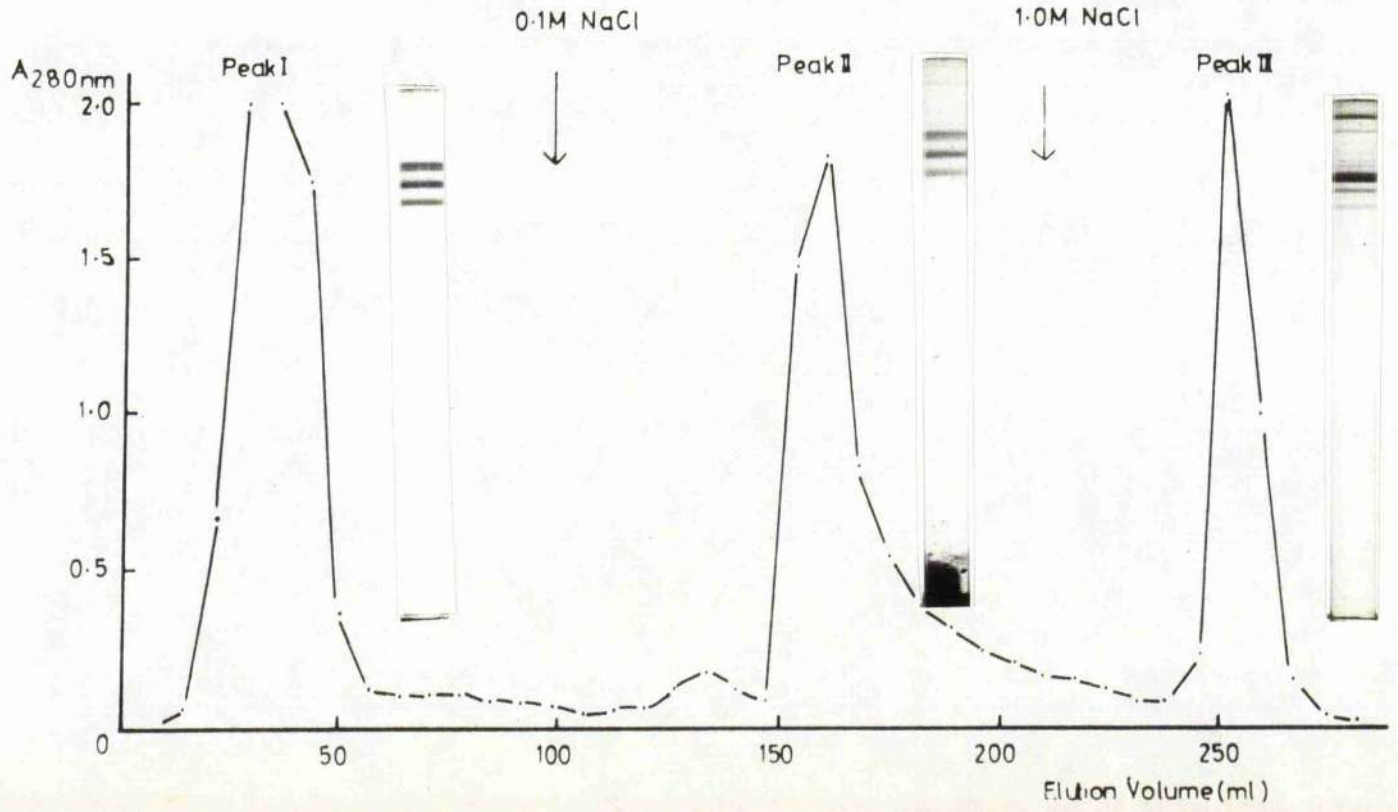

0·1M NaCl
1·0M NaCl
A280nm
2·0
1·5
1·0
0·5
0
Peak I
Peak II
Peak III
50
100
150
200
250
Elution Volume (ml)

Figure 9: Tests on Fibrinogen Purified from Human Plasma

a) plasminogen test: 5% SDS-PAGs of reduced samples from

i) Peak I
ii) Peak II
iii) Peak III

from DEAE-Cellulose chromatography

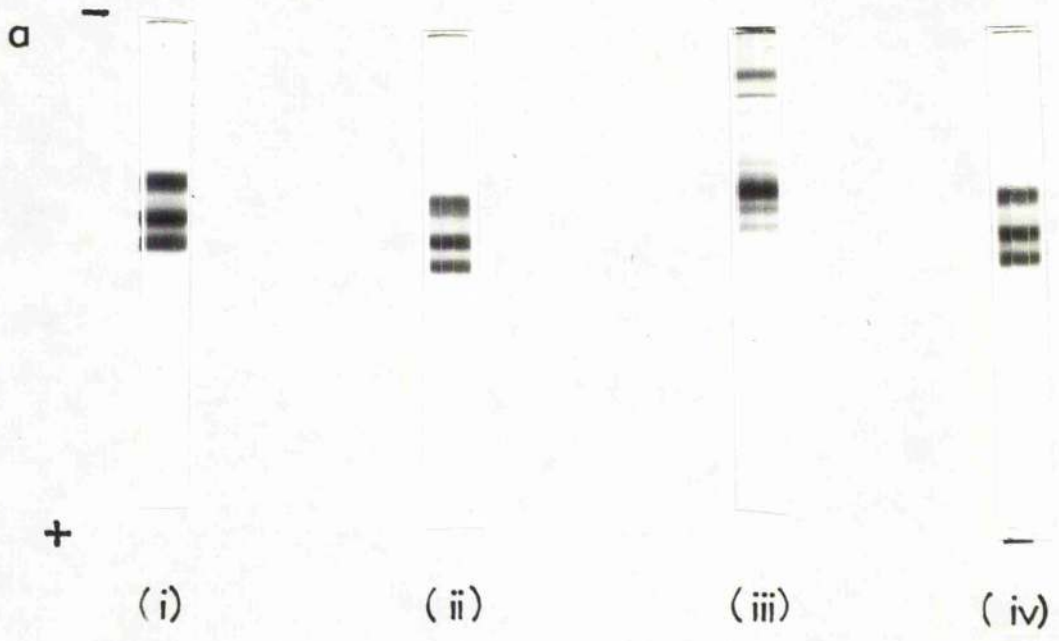
iv) standard fibrinogen

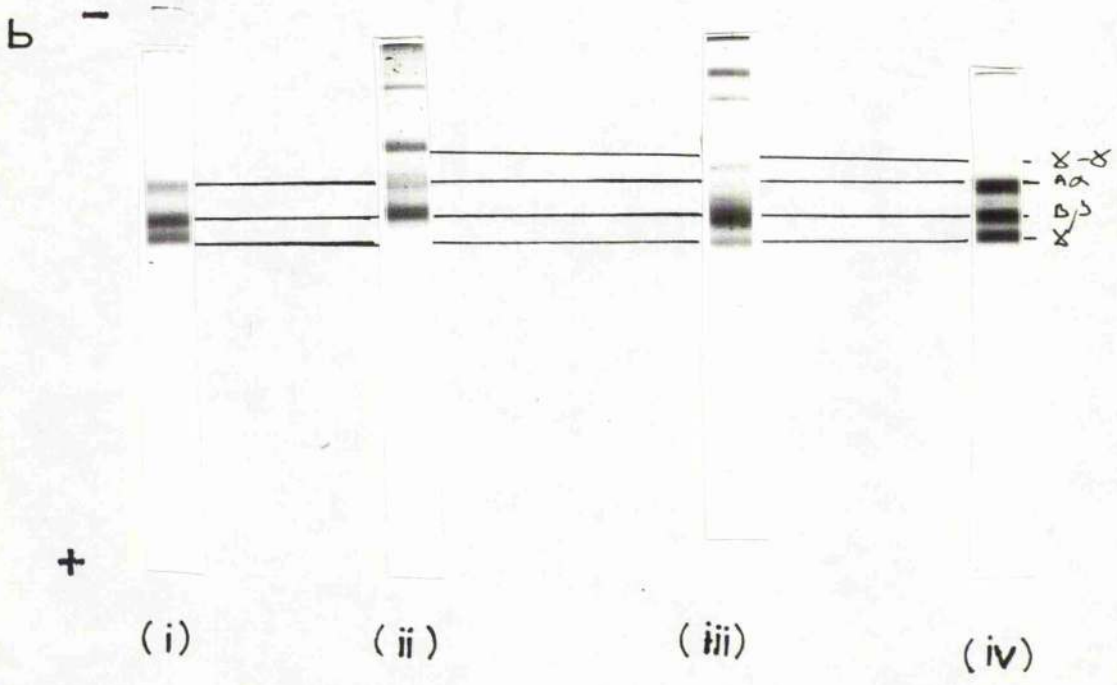
b) Factor XIII test: 5% SDS-PAGs of reduced samples from

i) Peak I
ii) Peak II
ii) Peak III

from DEAE-Cellulose chromatography

iv) standard fibrinogen


a
−
+
−
(i)
(ii)
(iii)
(iv)


b
−
+
(i)
(ii)
(iii)
(iv)

Peak	% Yield ± A.D.
I	51.1 ± 9.8
II	28.6 ± 8.7
III	17.7 ± 6.2

Table IV: Percentage Yield of Protein from DEAE-Cellulose Chromatography of Human Plasma.

The recovery of Peak I fibrinogen from the DEAE-Cellulose column was circa 1 mg fibrinogen for every ml starting plasma. As the concentration of fibrinogen in plasma is 3 mg/ml efforts were made to increase the recovery of fibrinogen from the purification. The supernatants from the 25% saturated and 23% saturated ammonium sulphate precipitation were examined by immunoelectrophoresis after dialysis against Tris/NaCl buffer pH 8.6 (Figure 10). A precipitate formed when the 25% saturated ammonium sulphate supernatant was left at $4^{o}C$ overnight. This precipitate was re-dissolved in Tris/NaCl buffer pH 8.6 and dialysed against the same buffer before being examined by immunoelectrophoresis (Figure 10). It can be seen that very little fibrinogen is lost in the 23% saturated ammonium sulphate wash while a considerable amount of protein is removed. The 25% saturated ammonium sulphate supernatant contains only a small proportion of fibrinogen. However the precipitate which formed at $4^{o}C$ was found to contain a considerable portion of fibrinogen. When this re-suspended precipitate was examined by SDS-

Figure 10: Immunoelectrophoresis of Samples from Ammonium Sulphate Fractionation of Human Plasma

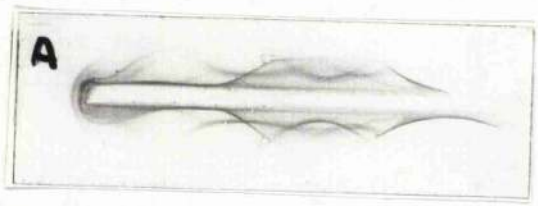
a) & d) test sample: supernatant from 23% saturated ammonium sulphate precipitation

b) & e) test sample: supernatant from 25% saturated ammonium sulphate precipitation

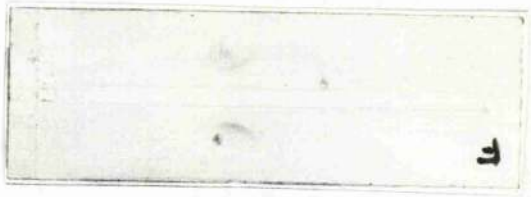
c) & f) test sample: resuspended 24 h precipitate from 25% saturated ammonium sulphate precipitation

a) b) & c) antibody: antiserum to whole human serum

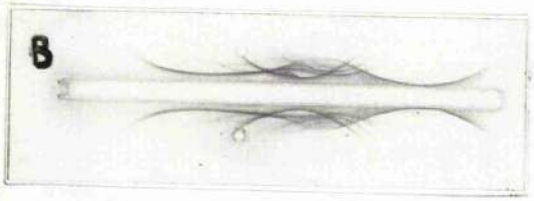
d) e) & f) antibody: antiserum to human fibrinogen



a



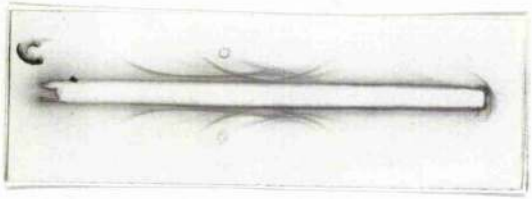
d



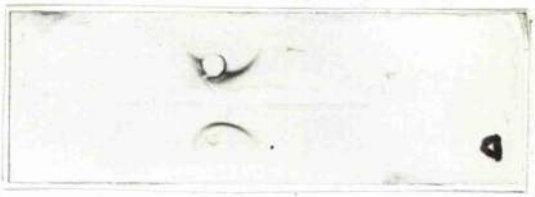
b

e

ANODE



c



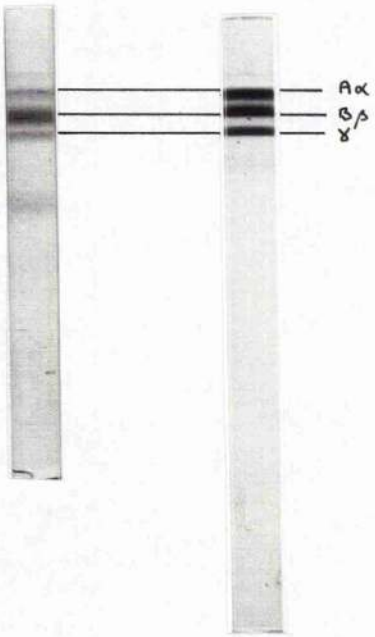
f

Figure 11: Electrophoresis of the Resuspended 24 h precipitate from 25% Saturated Ammonium Sulphate Precipitation

10% SDS-PAGs of -

i) resuspended 24 h precipitate from 25% saturated ammonium sulphate precipitation

ii) standard fibrinogen


Aα
Bβ
γ
(i)
(ii)

PAGE the fibrinogen present was found to be badly degraded (Figure 11). Therefore throughout the fractionation procedure there was no step at which a significant increase in yield of high quality fibrinogen could be easily obtained.

This rapid purification method enabled a high quality fibrinogen to be obtained in less than 36 h. The preparation was, as far as could be detected, free from Factor XIII and plasminogen. Fibrinogen prepared by this method, unless otherwise stated, was used in all experiments.

3.3. CROSS LINKING OF FIBRINOGEN

Fibrinogen was cross-linked using dimethyl suberimidate dihydrochloride (DMS) (Figure 12) a bifunctional agent of length 1.10nm which reacts with the free amino groups of proteins. Dimethyl suberimidate will introduce cross-links of 0.97 nm length (Hadju et al., 1976).

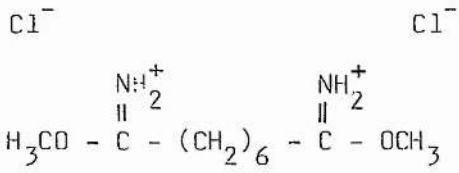


Figure 12: Structure of Dimethyl Subermidate Dihydrochloride

To obtain good detection by staining on SDS-PAGs at a suitable

sample loading a final protein concentration of 1 mg/ ml was considered optimal. A suitable concentration of DMS which gave predominantly intra-molecular rather than inter-molecular cross-linking was sought.

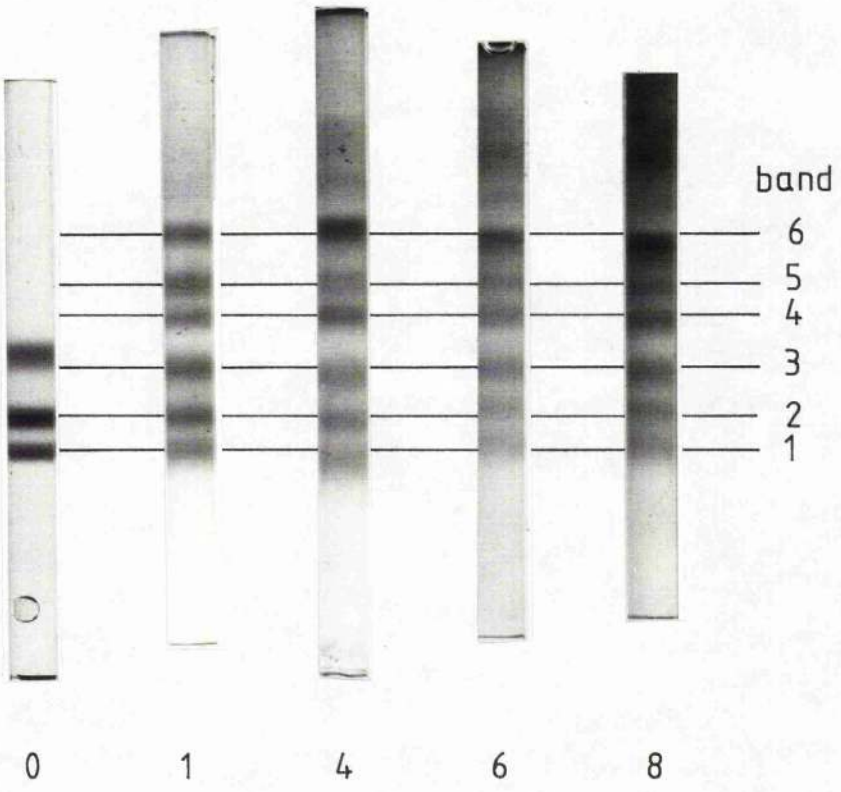
3.3.1. Effect of DMS Concentration on the extent of Cross-Linking

A series of fibrinogen samples, incubated with different concentrations of DMS for 1 h at 30°C, were examined by SDS-PAGE. From densitometer scans of reduced samples it was possible to estimate the extent of the intra and inter-molecular cross-linking. At DMS concentrations of 4 mg/ml and above there was extensive inter-molecular cross-linking. Cross-linking was apparent at DMS concentrations of 0.25 mg/ ml but at 1 mg/ml DMS the three cross-linked intermediates with molecular weights less than the intact molecule (Table V) and the three reduced chains of fibrinogen were all present in detectable quantities as well as the completely cross-linked molecule (Figure 13). This concentration of DMS of 1 mg/ml was used in further experiments.

Protein Band	Apparent Molecular Weight	N-Terminal Amino-acids	Probable Chain Composition
1	46,000	Tyr	γ
2	55,000	-	$B\beta$
3	70,000	Ala	$A\alpha$
4	95,000	Tyr	γ,γ or $B\beta,\gamma$
5	120,000	Ala, Tyr	$A\alpha,\gamma$
6	155,000	Ala, Tyr	$A\alpha, B\beta, \gamma$

Table V: Molecular Weights and Chain Composition of the Bands on SDS-PAGs of Reduced Samples of Cross-Linked Fibrinogen

Figure 13: Effect of DMS Concentration on the extent of Cross-Linking 4% SDS-PAGs of reduced cross-linked fibrinogen


band
6
5
4
3
2
1
0
1
4
6
8
DMS CONCENTRATION (mg/ml)

3.3.2. Effect of Calcium/EGTA on Cross-linking

Fibrinogen was cross-linked with DMS during incubation with either 2 mM $CaCl_2$ or 2 mM EGTA. Samples from each incubation were reduced and examined by SDS-PAGE alongside a control fibrinogen. When densitometer scans of the three preparations were compared (Figure 14) no gross differences in the pattern of cross-linking could be seen. Thus DMS is unable to detect a conformational change in the fibrinogen molecule in the presence and absence of calcium.

3.3.3. N-Terminal Analysis

The chains of reduced cross-linked fibrinogen, separated by SDS-PAGE, were extracted and N-terminal analysis of each extract performed. The chains thought to be present in each band are shown in Table V. The identification is tentative as the $B\beta$-chain has a blocked N-terminal and cannot be identified by dansylation and the apparent molecular weights of the cross-linked intermediates are less than the sum of the constituent chains. The band with the highest molecular weight which is less than the full molecule is thought to be the 'half-molecule' consisting of one of each of the three chains of fibrinogen, $A\alpha$, $B\beta$ and γ. The other two bands are composed of γ-γ or $B\beta$-γ and $A\alpha$-γ chains. There is no indication of the presence of an $A\alpha$-$A\alpha$ chain dimer.

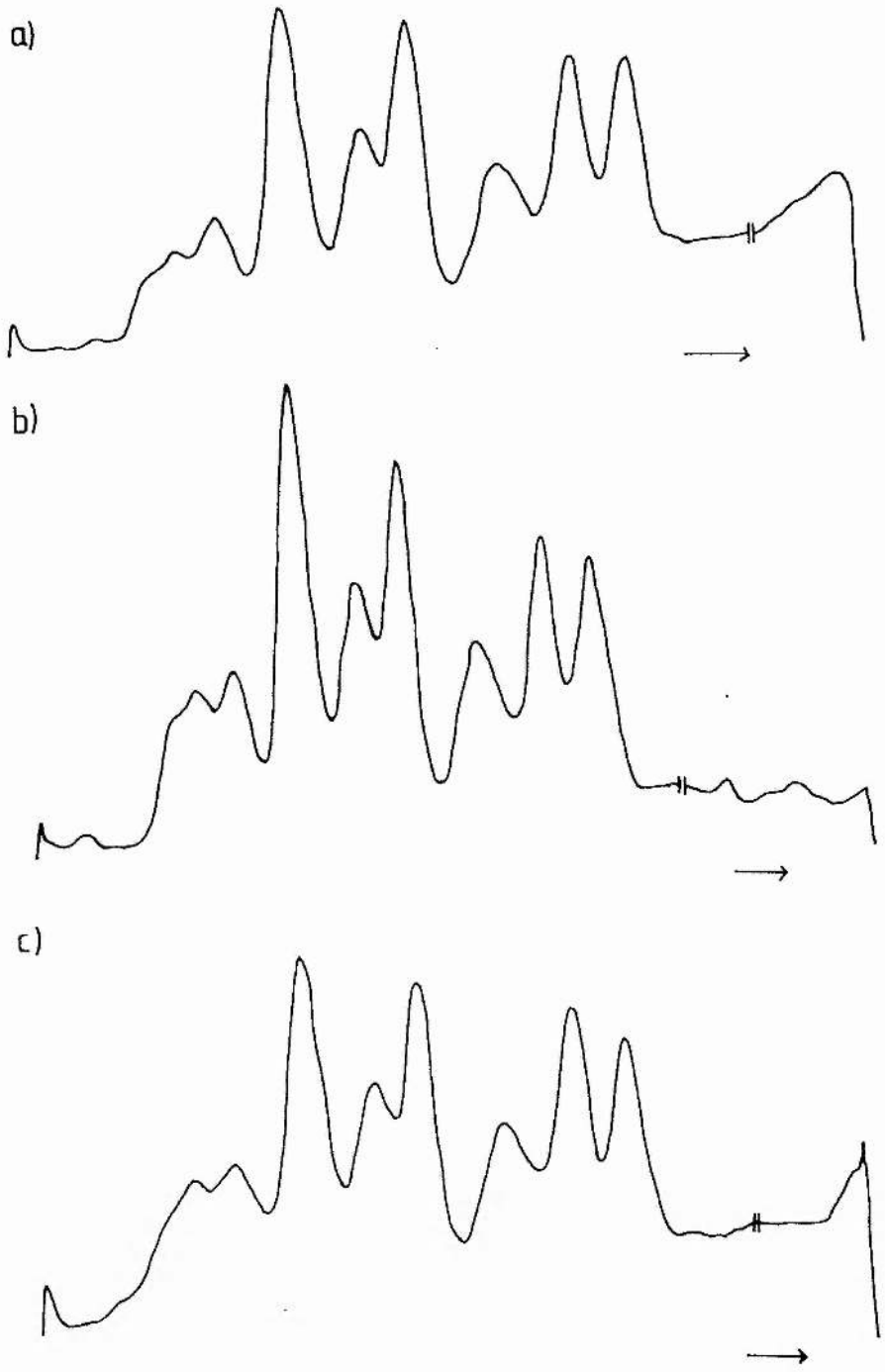


Figure 14: Effect of Calcium/EGTA on Cross-Linking of Fibrinogen

Densitometer scans of 4% SDS-PAGs of fibrinogen cross-linked in -

a) 0.05 M Tris pH 7.5 made 0.05 M with respect to NaCl.

b) the same buffer made 2 mM with respect to $CaCl_2$.

c) the same buffer made 2 mM with respect to EGTA.

The reaction of fibrinogen with DMS gives rise to cross-linking predominantly within the half fibrinogen molecule. There is little association between the two half-molecules as can be deduced from the absence of any major bands between those of the half-molecule and the full fibrinogen molecule of reduced samples run on SDS-PAGs. From this it can be concluded that band four (Figure 13) is most probably a γ-Bβ complex rather than a γ-γ dimer as the latter would favour the presence of bands between the half-molecule and the fibrinogen molecule.

No change in the cross-linking pattern was detected on SDS-PAGs when fibrinogen was cross-linked in the presence of calcium or EGTA. The results of N-terminal analysis indicate that DMS does not form cross-links between the two halves of the fibrinogen molecule therefore no information regarding major changes in conformation, which might arise from changes in the number of occupied calcium binding sites, can be obtained from cross-linking studies using DMS.

3.4. ELECTROPHORESIS OF ^{45}Ca-FIBRINOGEN

Lawrie & Kemp (1979) found that, in the presence of calcium, fibrinogen, when examined by SDS-PAGE after reduction at 20^{o}C gave four bands. The extra band, not normally found with reduced fibrinogen, had a mobility greater that the γ- chain and corresponded to a decrease in the size of the γ-chain peak on densitometer

scans when compared to fibrinogen reduced at 100°C. They concluded that calcium formed an intra-chain bridge at the carboxyl-terminal of the γ-chain.

This work was repeated using radioactively labelled calcium. On 5% SDS-PAGs reduced fibrinogen gave four bands with molecular weights between 40,000 and 70,000 although not all the fibrinogen was in the reduced form. The extra band had a mobility greater than that of the γ-chain. A gel, run under identical conditions but not stained was sliced and the slices tested for the presence of ^{45}Ca-ions. No radioactivity was detected in any of the slices. Non-reduced fibrinogen samples were run on 4% SDS-PAGs and similarly treated. Again no radioactivity was detected although there was radioactivity present in the sample applied.

3.5. PLASMIN DIGESTION OF ^{45}Ca-FIBRINOGEN

The concentration of calcium in ^{45}Ca-fibrinogen and plasmin-digested ^{45}Ca-fibrinogen preparations was estimated by measuring the amount of labelled calcium present and assuming proportionality between labelled and unlabelled ligand in the initial and final solutions.

From the results of four separate labelling experiments the molar ratio of calcium to fibrinogen was 3.1 $\pm$ 0.3. The plasmin digested samples were found to contain 35-40% less calcium than the corresponding intact samples. These results suggested that limited digestion of fibrinogen led to a reduction in the number of high affinity binding sites in the molecule and the probable involvement of the carboxyl

terminal of the A α -chains.

To obtain more quantitative data these experiments were repeated utilizing the flow dialysis apparatus. The results of these further experiments and subsequent work in preparing 'calcium-free' solutions suggest that the results obtained above are artefacts. The buffers used were not pre-treated in any way and contained both NaCl and $MgCl_2$, both of which were later found to contain significant levels of contaminating calcium. This would lead to an underestimation of the amount of calcium bound. Also, the excess of magnesium ions over calcium ions was probably not sufficient to prevent the low affinity binding of calcium to fibrinogen which affects the amount of calcium bound to the plasmin-digested fibrinogen.

3.6. FLOW DIALYSIS

3.6.1. Preliminary Experiments

Initial experiments were performed to determine the membrane properties with respect to calcium and to confirm the equilibration volume required, given by Colowick & Womack (1969) as four to five times the lower chamber volume.

The first experiments were carried out using a flow cell kindly lent by Dr. J. Ingledew who recommended the use of a double membrane for optimum performance. This flow cell had a lower chamber volume of 1 ml and an upper chamber volume of 0.5 ml.

The results of dialysis experiments performed in the absence of protein indicated that a much longer equilibration time was necessary than that suggested by Colowick & Womack. From these initial experiments the disadvantages of such a small working volume in the upper compartment were also evident. Only a small number of additions could be made and, because of the small initial volume, the dilution effect on the isotope was noticeable as a drop in the plateau level of radioactivity in the effluent. In addition, high flow rates could not be used as this resulted in back-pressure which adversely affected the rate of dialysis and hence the speed of equilibration.

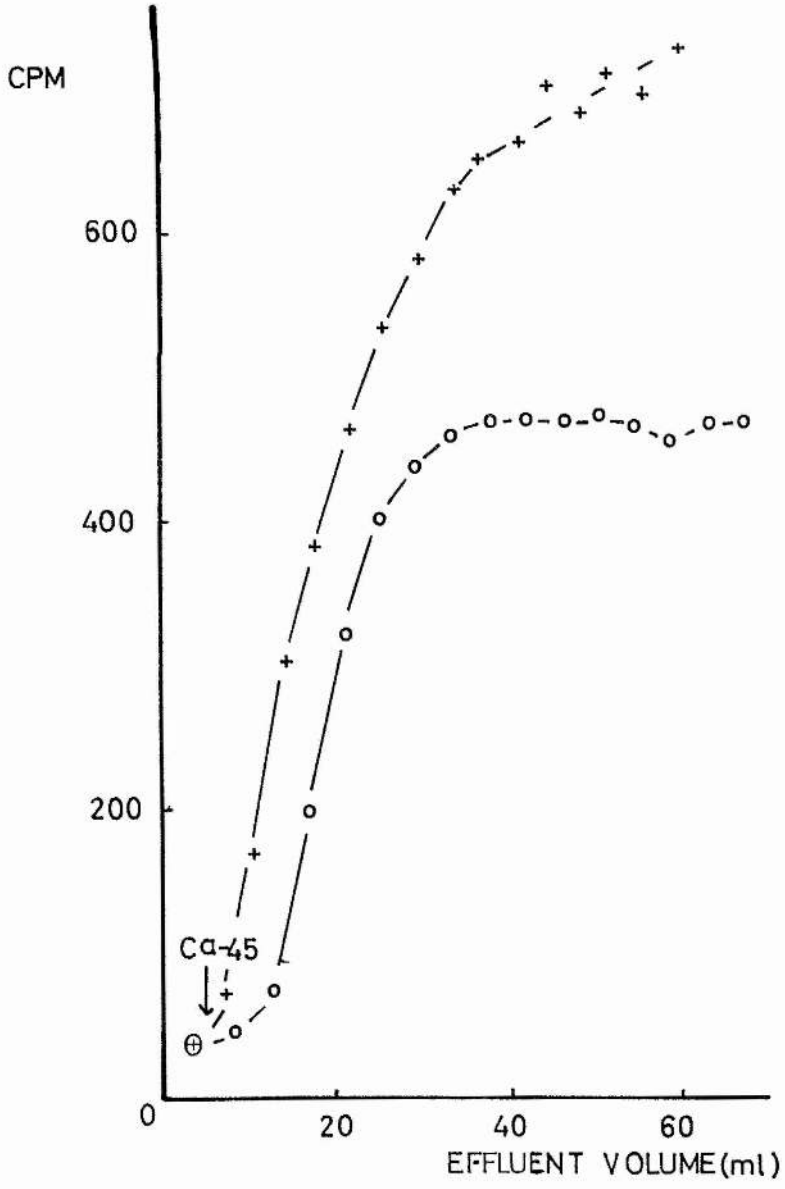
The larger flow cell obtained from Bel Art Products Ltd. was used in further equilibrium dialysis experiments, similar to those performed with the small flow cell, in which fibrinogen was omitted from the upper compartment. Dialysis sheets designed specifically for use with the flow cell were found to require eight times the lower chamber volume, twice the expected buffer volume, to reach equilibrium while, with Sigma and Visking double membranes, equilibrium had not been reached after sixteen times the lower chamber volume of buffer had passed through the cell (Figure 15).

A possible explanation of the slow equilibration times was low affinity binding of calcium to the dialysis membranes. In order to ascertain if this was so each of the three

Figure 15: Preliminary Flow Dialysis Experiments. The Equilibration of Membranes

(O —— O) Single Dialysis Sheet (Bel-Art Products)

(+ —— +) Double Sigma Membrane


CPM
600
400
200
0
Ca-45
20
40
60
EFFLUENT VOLUME(ml)

membrane types was tested by adding 10 µL of 1 mM $^{40}CaCl_2$ to the upper chamber once the system had reached equilibrium. In each case there resulted a peak of radioactivity in the effluent before the level of radioactivity returned to a steady-state at a slightly lower level than that before the addition (Figure 16). The addition of non-radioactive ligand should not have affected the steady-state as the concentration of isotope in the effluent is dependent only on the fraction of substrate free in the upper chamber and not on the total concentration of substrate. The appearance of a pulse of radioactivity into the effluent on the addition of $^{40}CaCl_2$ suggested that calcium ions were binding to the membrane. Pre-treatment of the membranes with bicarbonate and EGTA did not eliminate this response.

In order to nullify the effects of any charged groups on the membrane which might be interfering with dialysis the Sigma and Visking membranes were acetylated. The dialysis sheets which are a single layer could not easily be acetylated. This did not significantly alter the equilibration time but did reduce the level of radioactivity in the eluent (Figure 17).

Single Sigma and Visking membranes were then tried. These systems were found to equilibrate within the expected period and did not give a peak of radioactivity on the addition of 10 µL of $^{40}CaCl_2$. However the use of single membranes resulted in a significant increase in the plateau level of radioactivity

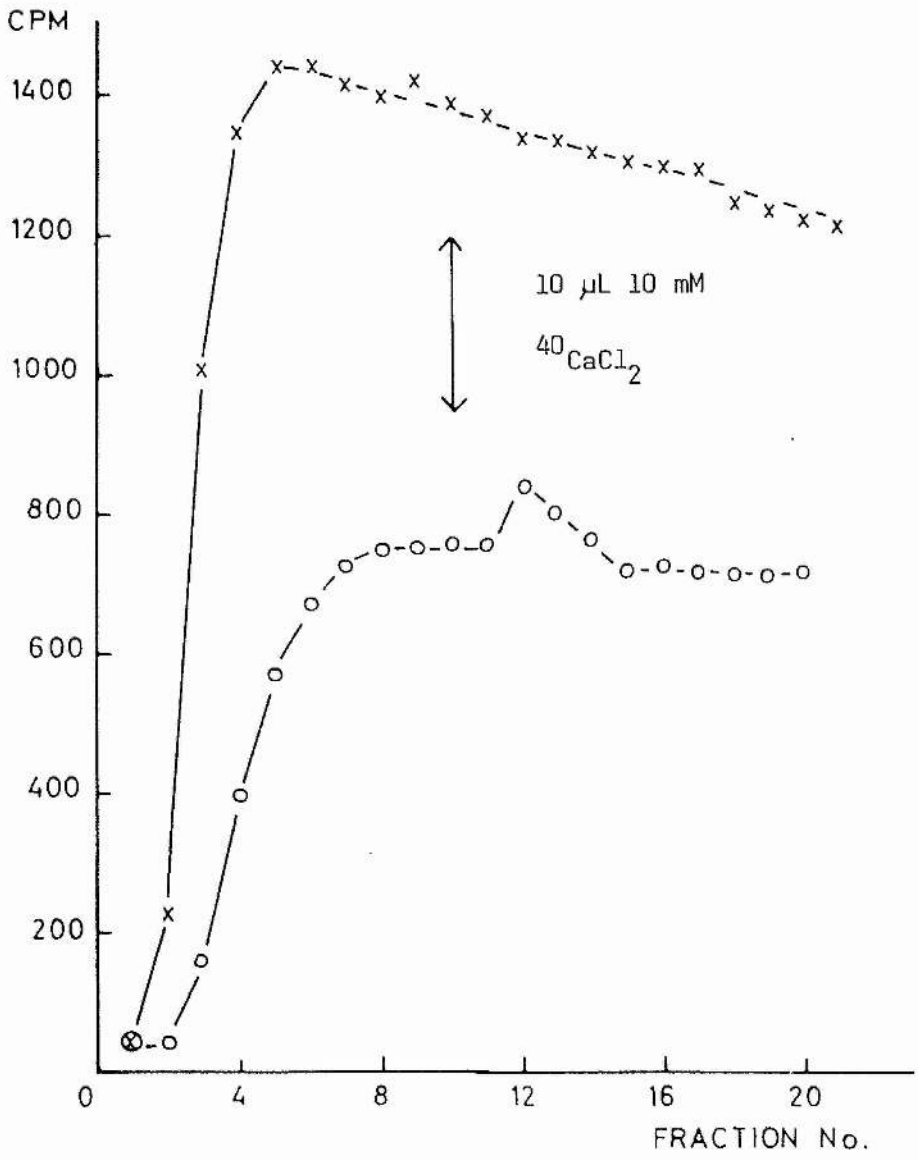


Figure 16: Flow Dialysis Preliminary Experiments

Additions of $^{40}CaCl_2$ to the Flow Cell after the Establishment of Equilibrium

(X——X) Single Sigma Membrane

(O——O) Double Visking Membrane

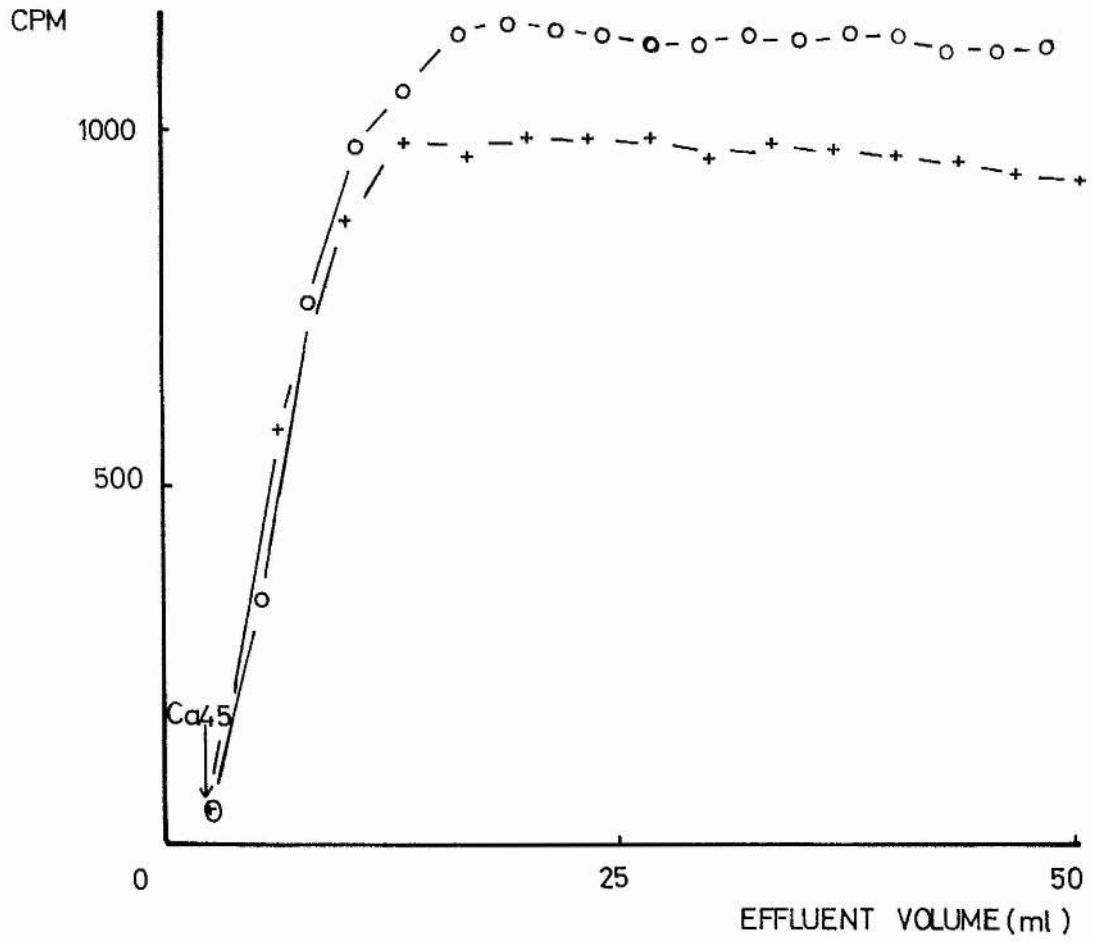


Figure 17: Flow Dialysis Preliminary Experiments

The Effect of the use of Acetylated Membranes

(O —— O) Single Visking Membrane - non-acetylated

(+ —— +) Single Visking Membrane - acetylated

in the eluent. To reduce the amount of label lost dialysis was carried out using acetylated membranes. This should also have eliminated any artefacts which might arise from small quantities of calcium binding to the dialysis membrane.

As a result of these preliminary investigations acetylated single layer Sigma dialysis membranes were used in subsequent experiments.

Before the flow dialysis cell could be used for rate of dialysis it had to be modified to reduce the amount of calcium lost during the course of an experiment. This was done by placing the dialysis membranes between two perpex inserts thus reducing the surface area of the membrane exposed to the solvent. Figure 18 compares the elution profile of the modified with the un-modified cell in a protein- free assay. Using the insert there was a reduction by two-thirds, of the amount of calcium lost in the eluent without any change in the buffer volume required to reach steady-state.

3.6.2. Preparation of Calcium-Free Solutions

3.6.2.1. Atomic Absorption Spectrometry

The calcium concentrations in fibrinogen and buffer samples were measured by Atomic Absorption Spectrometry (AAS). To obtain accurate data care must be taken to ensure interference effects are minimised. The two types of interference most likely to

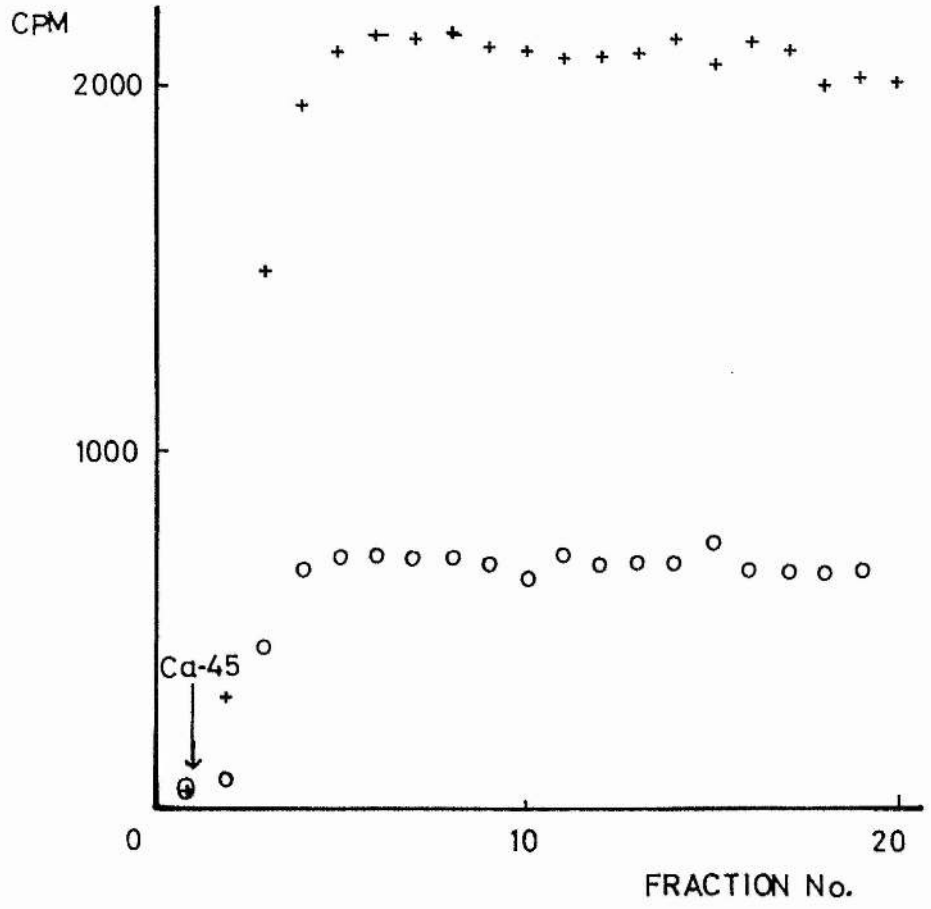


Figure 18: Modification of the Flow Dialysis Cell: The Effect of Modification on the Amount of Radioactivity in the Effluent

(O —— O) With perspex insert

(+ —— +) Without perspex insert

effect measurement of calcium in fibrinogen solutions are ionisation and matrix interference. The former occurs when hot flames, e.g. nitrous oxide/acetylene, are used to measure elements, such as calcium, with relatively low ionisation potentials. It can be overcome by the addition of another easily ionised element, such as sodium, and will result in an increase in absorbance of the measured element (Figure 19a). Matrix interference can arise from differences in viscosity in samples and standards, which in turn affects the rate of nebulisation within the flame. However the effect of viscosity is negligible when samples are in dilute aqueous solution. But, because of these effects, it is of primary importance for standards and samples to have, as far as possible, the same chemical composition.

To ensure that the effects of interference were overcome the method of standard additions was used when determining the calcium composition of fibrinogen samples. For this a sample was split into a number of equal sized aliquots and different amounts of standard calcium solution were added to each. Finally all aliquots were diluted to the same volume giving a series of samples of constant protein concentration but varying calcium concentrations. When the absorbance readings were plotted against the final known calcium concentration of the samples a line parallel to that for buffer standards was obtained (Figure 19b). When the line is

Figure 19: Atomic Absorption Spectrometry

a) Ionisation Interference: its Elimination by the Presence of Sodium Ions

(□——□) Calcium measured in distilled H_2O

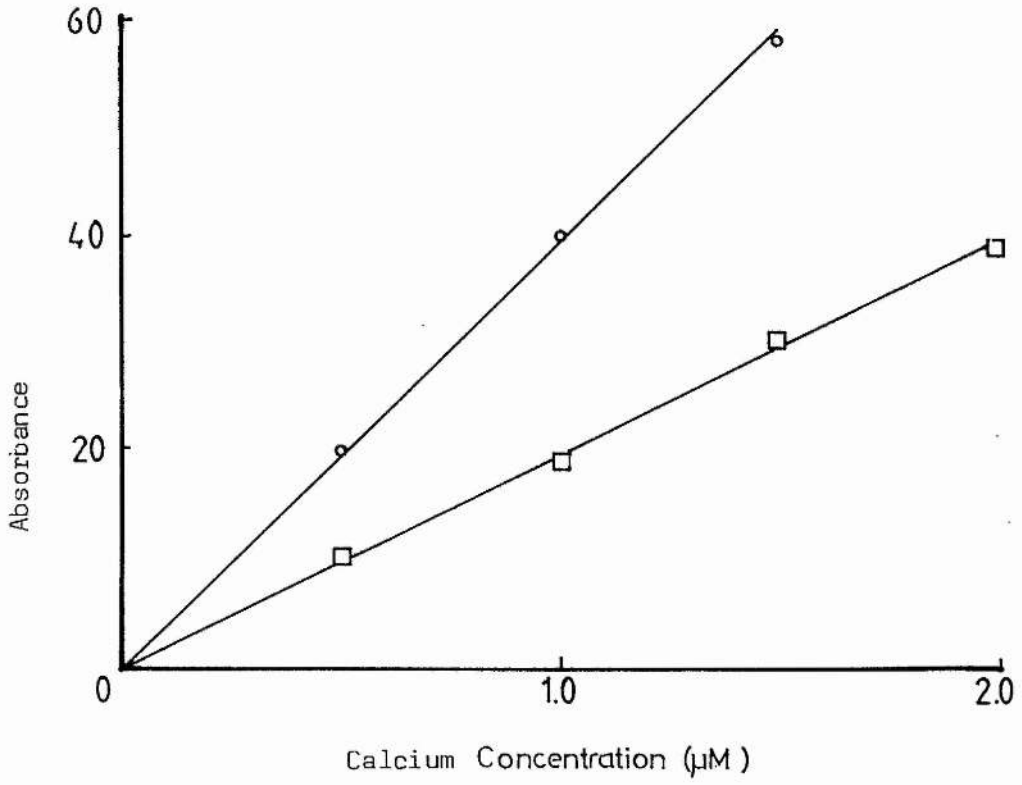
(○——○) Calcium measured in 0.05M Tris pH 7.5 made 0.15 M with respect to NaCl

b) The Method of Standard Additions

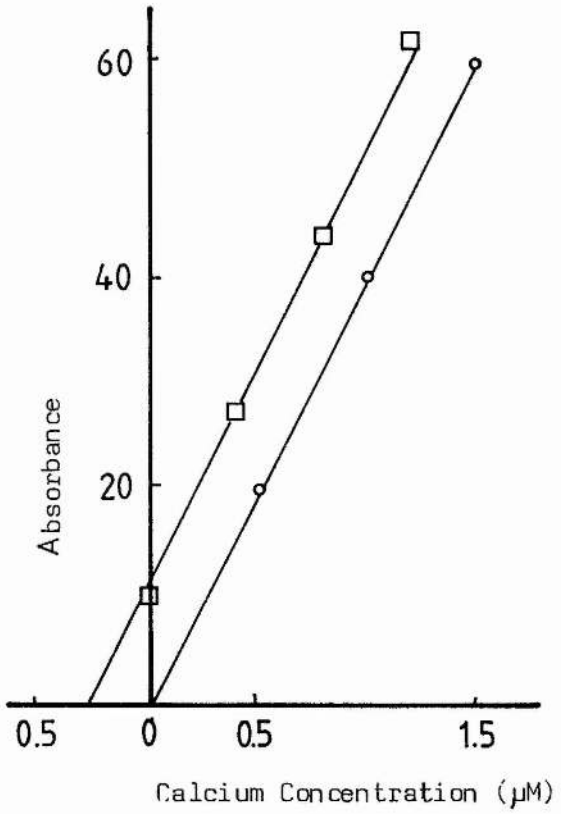
(□——□) Fibrinogen and standard calcium solutions

(○——○) Standard calcium solutions

a)


60
40
20
0
Absorbance
1.0
2.0
Calcium Concentration (μM)

b)


60
40
20
Absorbance
0.5
0
0.5
1.5
Calcium Concentration (μM)

extrapolated the point where it cuts the abscissa indicates the concentration of calcium corresponding to the fibrinogen sample.

3.6.2.2. Preparation of Calcium-Free Buffers

Buffer solutions were prepared by passing concentrated solutions of the individual buffer components through a Chelex-100 column and diluting these in appropriate ratios before use. The calcium concentrations of small samples of the diluted preparations were measured by AAS and the results are shown in Table VI. $MgCl_2$ solutions were not passed through the Chelex-100 column as the resin has a similar affinity for Ca^{2+}-ions and Mg^{2+}-ions therefore calcium would not be removed selectively.

Buffer	before Chelex-100 [calcium] (μM)	after Chelex-100 [calcium] (μM)
0.05 M Tris pH 7.5	1.9	1.0
0.15 M NaCl	3.75	-
0.05 M Imidazole pH 7.5	-	-
0.05 M Tris 0:15 M NaCl pH 7.5	5.0	1.8

Table VI: Calcium Concentrations of Buffer Solutions Measured by AAS

Values of (-) indicate calcium concentrations outwith the sensitivity of the instrument.

When 10 mM $MgCl_2$ was added to the Chelex-100 treated Tris/NaCl buffer the resulting solutions had levels of contaminating calcium in the region of 3.25 μM.

3.6.2.3. Preparation of Calcium-Free Fibrinogen

To remove calcium, fibrinogen was first dialysed against Tris/NaCl buffer and made 1 mM with respect to EGTA followed by dialysis against two changes of the same buffer from which EGTA was omitted. The dialysis tubing (Visking) was first washed in $NaHCO_3$/EGTA and rinsed extensively in distilled H_2O. When compared to similar samples dialysed in tubing which was washed only in distilled H_2O or had been acetylated before use no significant difference in the calcium/fibrinogen molar ratio was found. Small aliquots of protein were tested after each stage of dialysis (Table VII) and as expected there was a decrease in the calcium concentration of the solution. However after a third change of EGTA free buffer there is a slight increase in the calcium/fibrinogen ratio.

Dialysis Step	Dialysis Buffer	Calcium/Fibrinogen
1	0.05 M Tris 0.15 M NaCl 1 uM EGTA pH 7.5	7
2	0.05 M Tris 0.15 M NaCl pH 7.5	1.2
3	0.05 M Tris 0.15 M NaCl pH 7.5	1.2
4	0.05 M Tris 0.15 M NaCl pH 7.5	2.4

Table VII: Molar Ratio of Calcium to Fibrinogen in Fibrinogen Samples at Different Stages of Dialysis

3.6.3. Plasmin Digestion of Fibrinogen

Fibrinogen was digested by plasmin in the flow dialysis cell to determine if any high affinity calcium binding sites could be detected in those parts of the molecule first attacked by plasmin.

Figure 20a follows the progress of digestion of fibrinogen by plasmin taking place in the top chamber of the flow dialysis cell. Within two minutes of the addition of plasmin an appreciable portion of the Aα-chain had been digested to a fragment of apparent molecular weight 25,000. No intact Aα-chain was evident after 20 min. The loss of calcium from the system is followed in Figure 20b. In both protein digest and in the standard there was an initial drop in the level of radioactivity in the eluent on the addition of plasmin as a result of dilution. The plateau level of the buffer standard was 300 cpm higher than that in the fibrinogen experiments which indicated that ^{45}Ca-ions were bound to the protein molecules. The elution profiles from the fibrinogen digestion and the fibrinogen standard are parallel. This would suggest that there was no significant release of calcium when fibrinogen was digested by plasmin. If calcium was bound at the carboxyl terminal of the Aα-chains an increase in unbound calcium in the upper chamber would be expected on the action of plasmin. This increase in the concentration of unbound calcium would result in an increase in the amount of radioactivity in the eluent buffer. The increase would not take the form of a pulse of radioactivity but would be an increase in the plateau level measured in the eluent.

Figure 20: Digestion of ^{45}Ca-Fibrinogen in the Flow Dialysis Cell

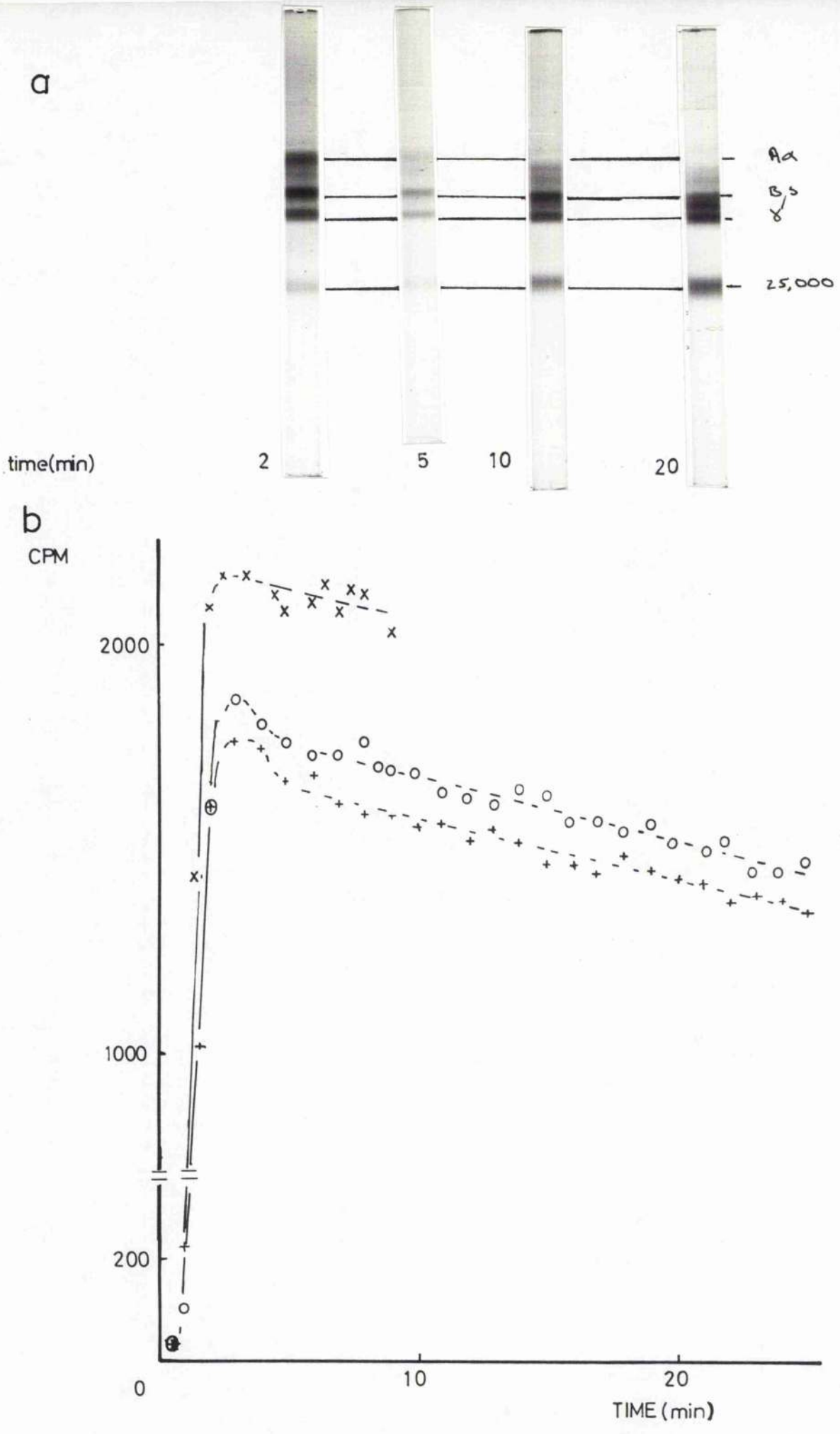
a) The extent of digestion of fibrinogen at various times after the addition of plasmin.

b) The loss of $^{45}Ca^{2+}$- ions from the flow cell during the digestion of fibrinogen by plasmin.

(X——X) Buffer standard

(O——O) Protein digest

(+ —— +) Protein standard


a
Aα
Bβ
γ
25,000
time(min)
2
5
10
20
b
CPM
2000
1000
200
0
10
20
TIME (min)

From these results and the results from the cross-linking experiments no evidence has been found to support the proposition that there is a high affinity calcium binding site at the carboxyl terminal of the A α-chains in fibrinogen.

3.6.4. Ligand Binding

Ligand binding experiments, using the flow dialysis technique and fibrinogen preparations with a high degree of integrity, were performed to obtain quantitative data regarding the number of high affinity calcium binding sites in fibrinogen.

Figure 21 shows the time course of the rate of dialysis of calcium in the presence and absence of fibrinogen. When excess calcium was added, in the presence of fibrinogen, the concentration of isotope in the effluent reached a maximum value, corresponding to 100% free calcium, which is close to that found for calcium alone. This indicated that only a small percentage of the total calcium is lost throughout the duration of the experiment. When the perspex insert was used, giving a reduced membrane surface area, the total calcium lost during the experiment was 7-8%. Without the insert 45/50% of the calcium was lost, a loss too great for meaningful data to be obtained.

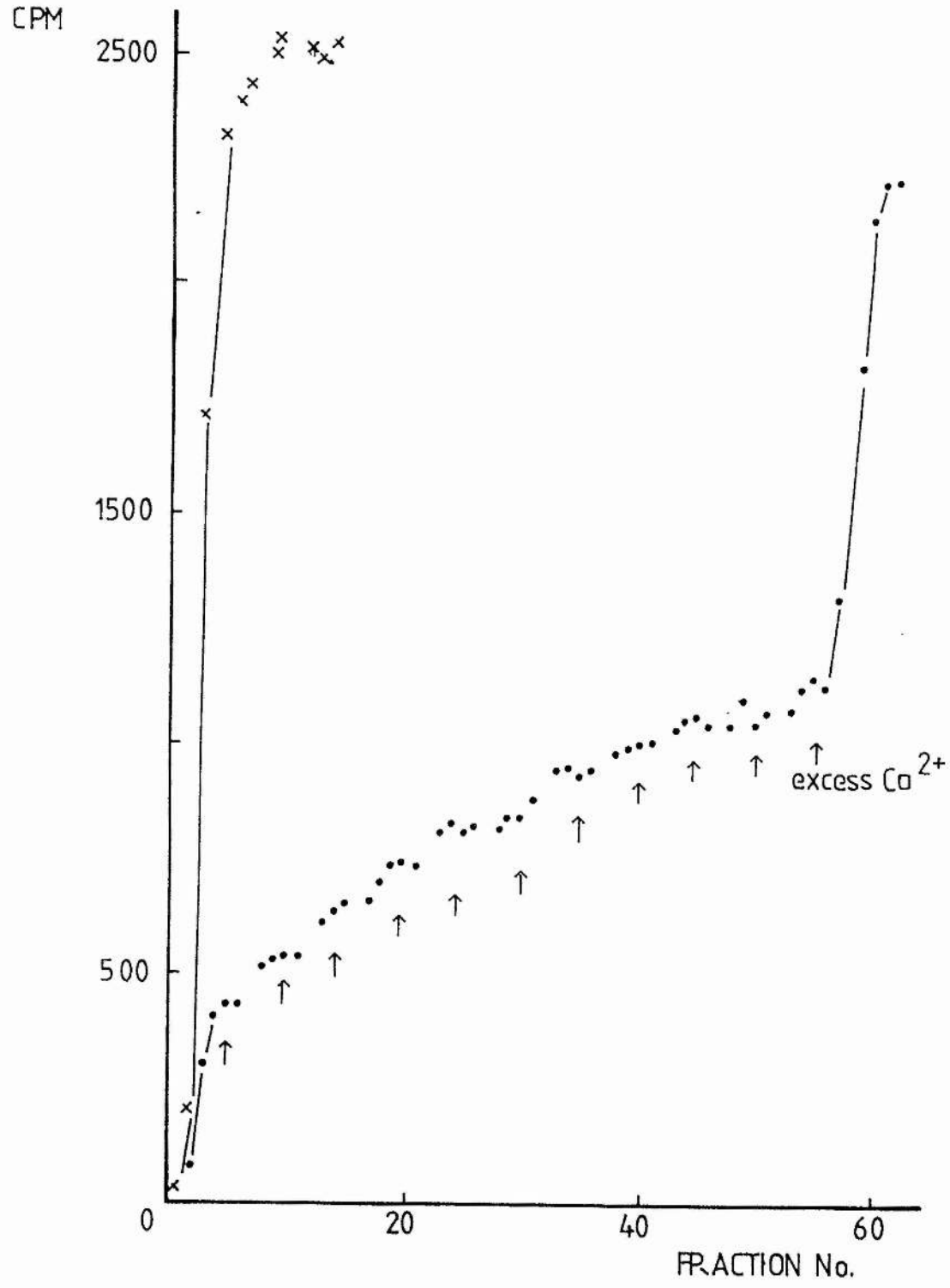
Figure 22 is a Scatchard plot drawn from data derived from rate of dialysis experiments. A linear plot was obtained indicating three equivalent calcium binding sites. The dissociation constant was

Figure 21: Ligand Binding - The Time Course of the Rate of Dialysis in the Presence and Absence of Fibrinogen

↑ Addition of $CaCl_2$

(• —— •) Presence of fibrinogen

(x —— x) Absence of fibrinogen


CPM
2500
1500
500
0
20
40
60
FRACTION No.
excess Co 2+

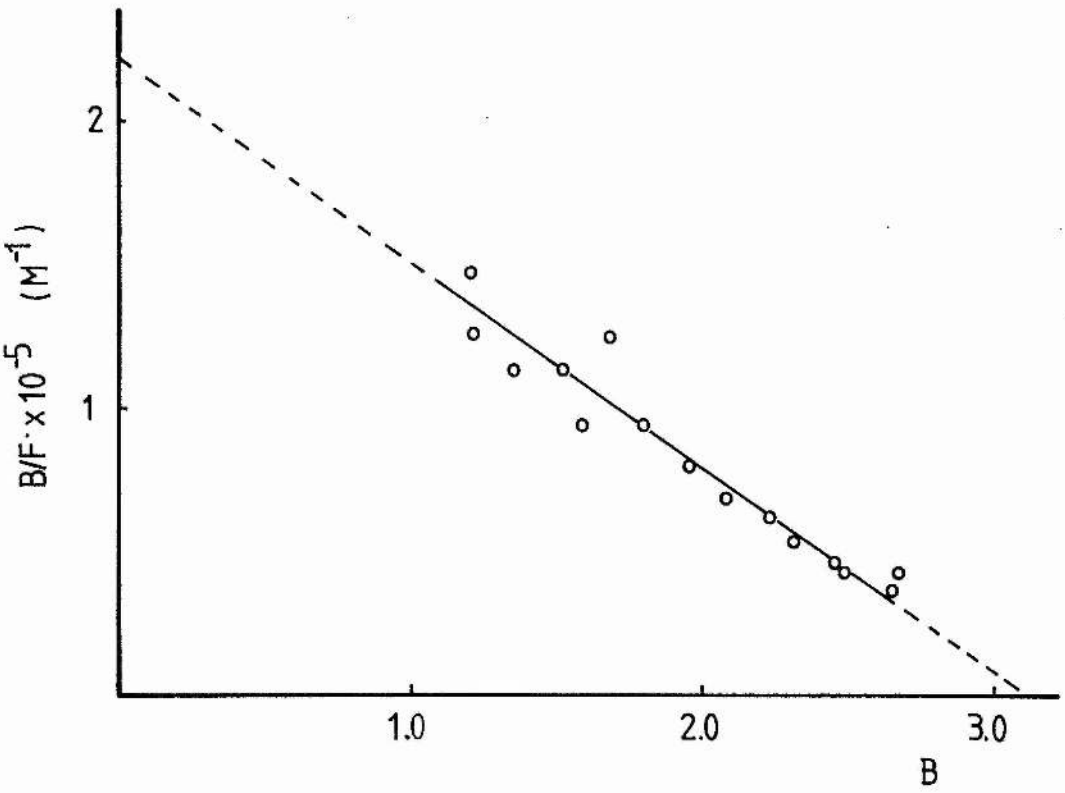


Figure 22: Ligand Binding - A Scatchard Plot Derived from Data Obtained from Rate of Dialysis Experiments

1.4×10^{-5}M as compared to 8.7×10^{-6}M reported by Lindsey et al. (1978). These results differ from those of Nieuwenhuizen et al. (1979) who reported two binding sites K_d 9.0×10^{-6}M and one binding site K_d 32×10^{-6}M.

However, data obtained in this way, using calcium-free fibrinogen, does not cover a sufficiently wide spectrum of values, in particular, the lowest "bound" value was never less than 1.1, yet those values between 0 - 1 calcium bound per fibrinogen molecule are crucial, particularly if the Scatchard plot is a curve. This problem arises from the relatively high concentrations of calcium in the "calcium-free" fibrinogen. In buffer systems using Tris and/or $MgCl_2$ the problem is exacerbated as neither of these compounds could be freed from contaminating calcium.

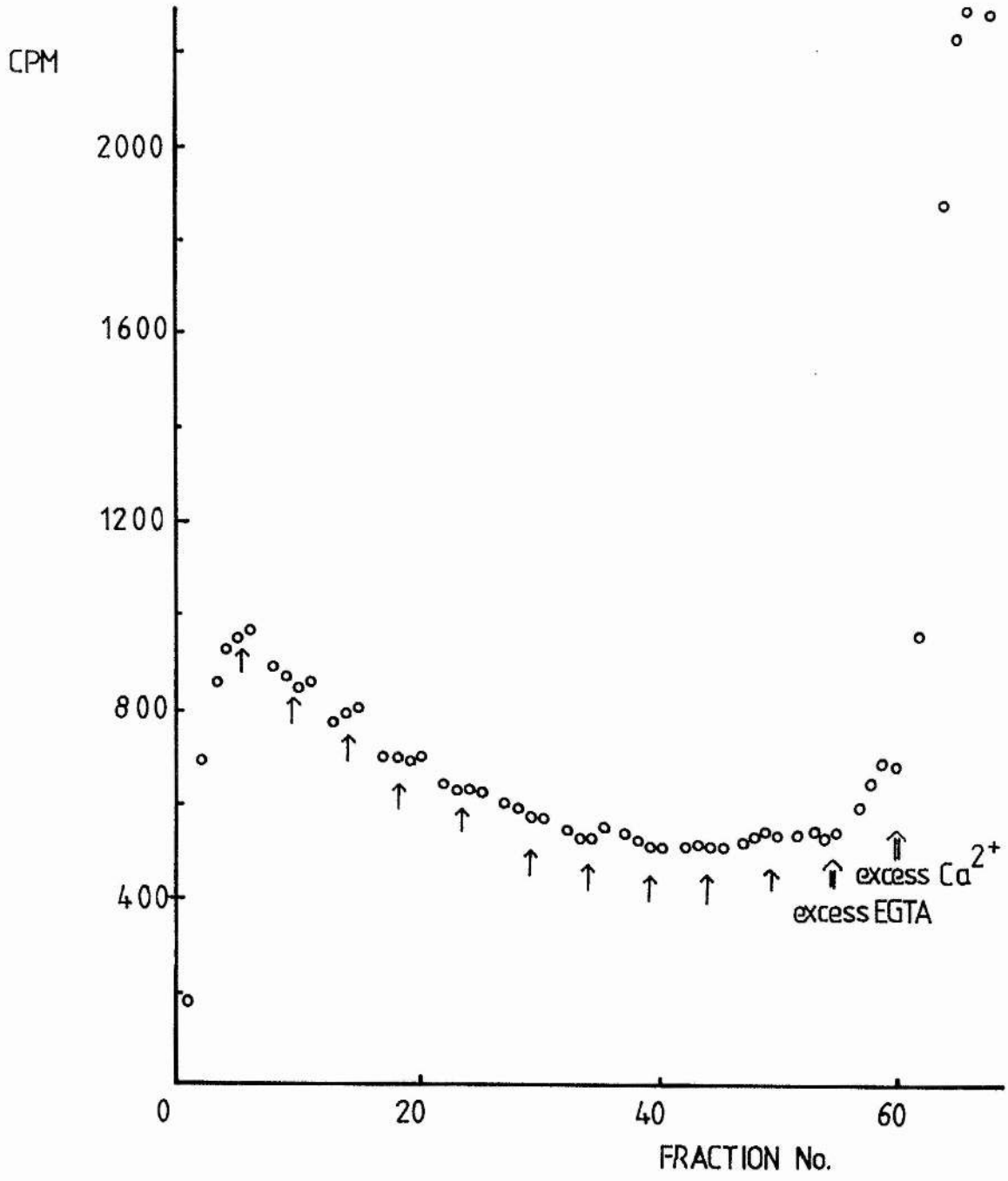
In view of these results and in order to overcome the need for a "calcium-free" fibrinogen it was decided to use reverse-ligand binding where calcium is removed from the system by the chelator EGTA.

3.6.5 Reverse Ligand Binding

Figure 23 follows the release of calcium from fibrinogen by increasing EGTA concentrations. There was an initial decrease of radioactivity in the eluent as the concentration of calcium in the eluent decreases because the EGTA-Ca^{2+}ion complex has a lower rate of dialysis than free calcium ions. The rates of dialysis of the EGTA-Ca^{2+}ion complex and calcium ions alone were calculated from

Figure 23: Reverse Ligand Binding - The Time Course of the Rate of Dialysis In 0.05 M imidazole/HCl pH 7.5 made 0.15 M with respect to NaCl.

↑ EGTA Additions


CPM
2000
1600
1200
800
400
0
20
40
60
FRACTION No.
excess Ca^{2+}
excess EGTA

the plateau levels obtained when excess EGTA and excess calcium were added to the flow dialysis cell. When the perspex insert was used less than 3% of the calcium was lost from the upper chamber while in the absence of the insert 9% of the ligand was lost during the experiment.

Figures 24/25 are plots of data derived from reverse ligand binding experiments in 0.05 M Imidazole pH 7.5 made 0.15 M with respect to NaCl. Figure 24 is the normal Scatchard plot of B/F against B, however, because of the sharp increase in B/F around B = 1, to accommodate all the points the scale on the ordinate axis has been broken. In order to obtain a continuous curve a plot of log (B/F) against B was made as is shown in Figure 25. When both curves are examined it is apparent that there is a marked increase in the value of B/F when the number of calcium ions bound to fibrinogen is about one. There is also a suggestion that the curve is falling back on itself at high concentrations of EGTA when the B/F values are highest.

Similar results were obtained when experiments were performed using 0.05 M Tris buffer pH 7.5 made 0.15 M with respect to NaCl and 10 mM with respect to $MgCl_2$ (Figure 26/27). The point of inflection is at a lower value of B because of the presence of magnesium and the falling back of the curve at high B/F values is more obvious.

The shape of the curve can be interpreted in a number of ways. The most obvious is to assume the presence of different groups of binding

Figure 24: Reverse Ligand Binding - In the Absence of Magnesium:

A Scatchard Plot Derived from Data
Obtained from Rate of Dialysis Experiments

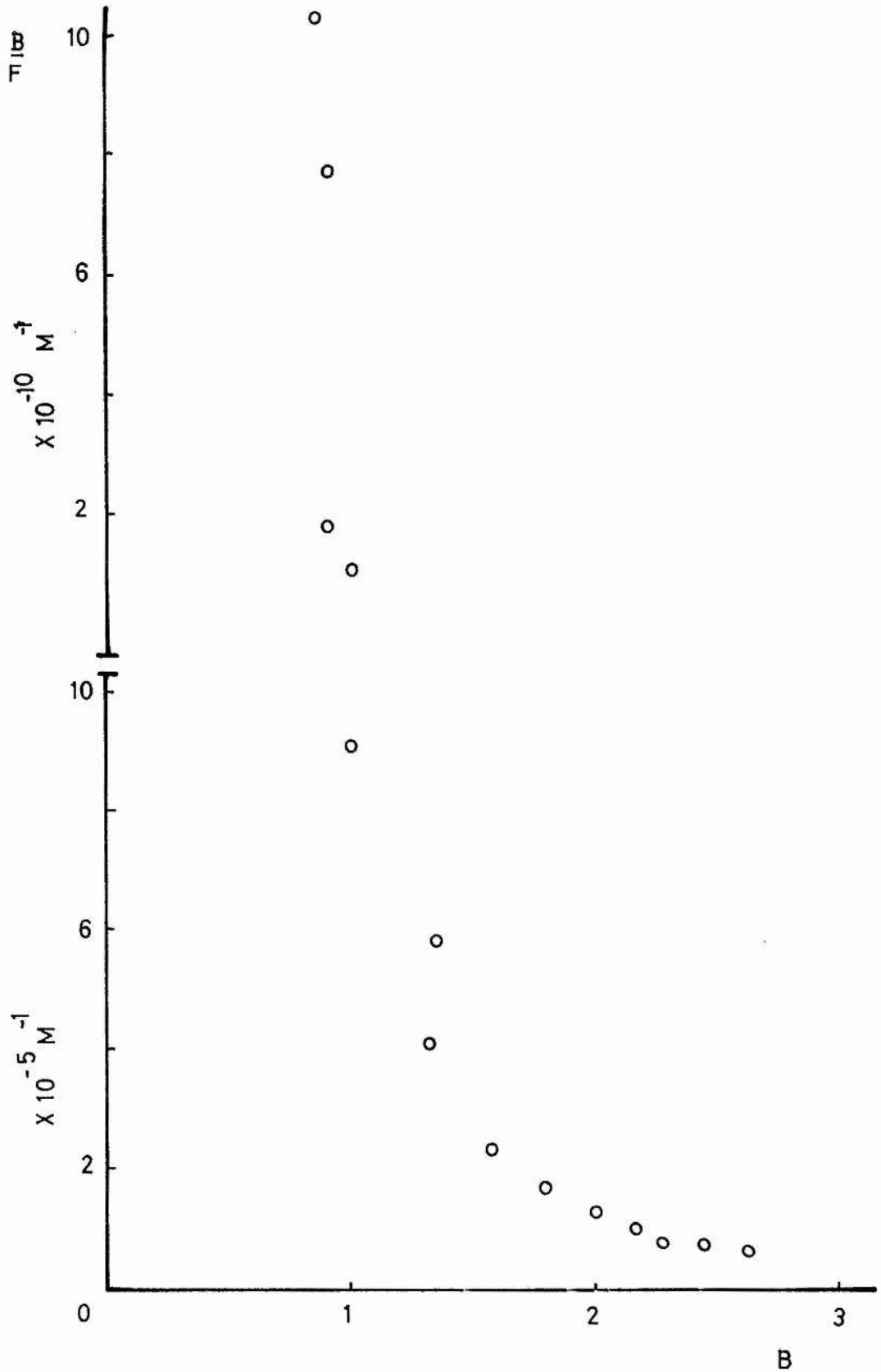

F I B
10
6
2
X 10^-10 M^-1
10
6
2
X 10^-5 M^-1
0
1
2
3
B

Figure 25: Reverse Ligand Binding - In the Absence of Magnesium. A Linear-Log Plot of Scatchard Data Obtained from Rate of Dialysis Experiments

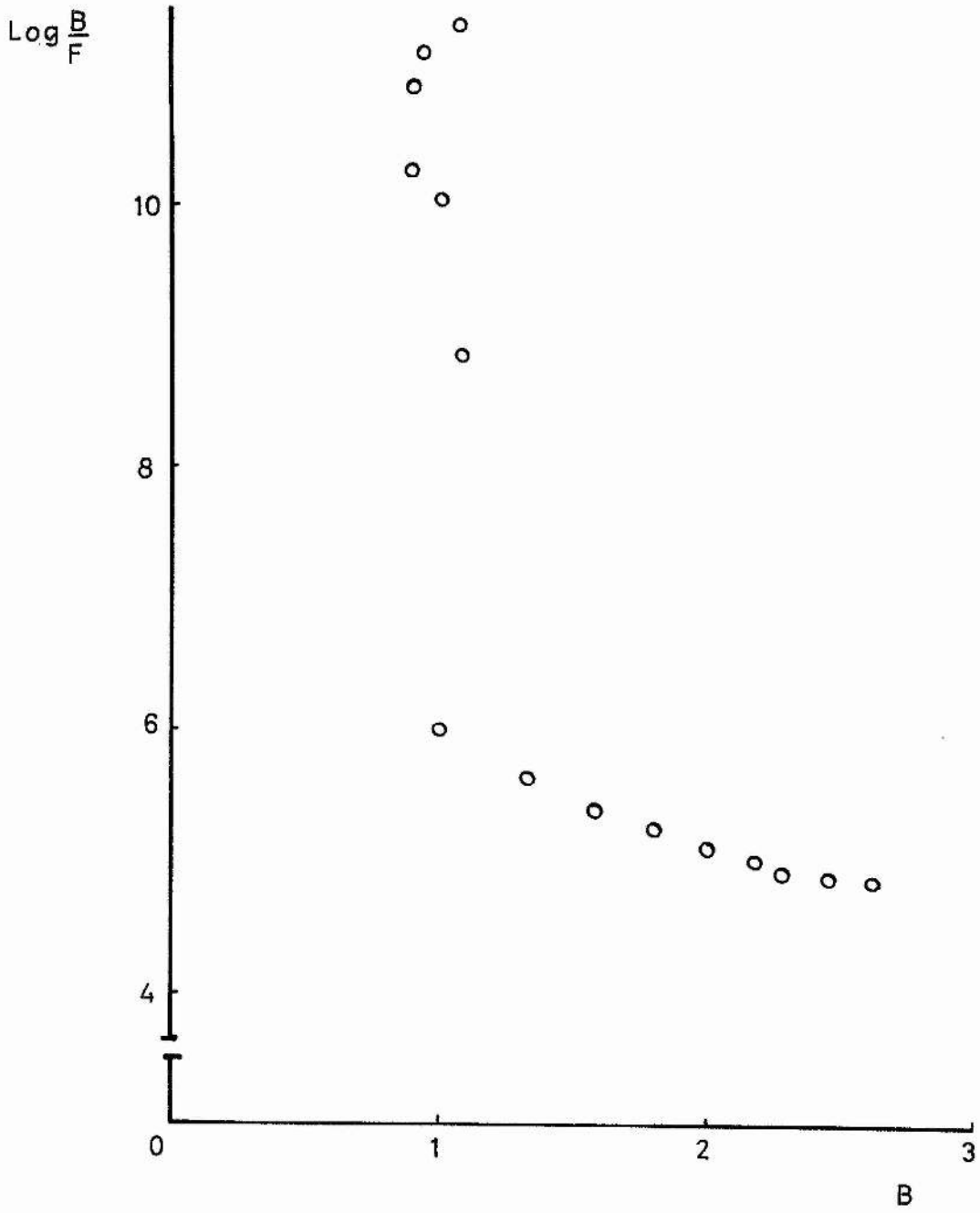

Log B/F
10
8
6
4
0
1
2
3
B

Figure 26: Reverse Ligand Binding - In the Presence of 10 mM $MgCl_2$: A Scatchard Plot Derived from Data Obtained from Rate of Dialysis Experiments

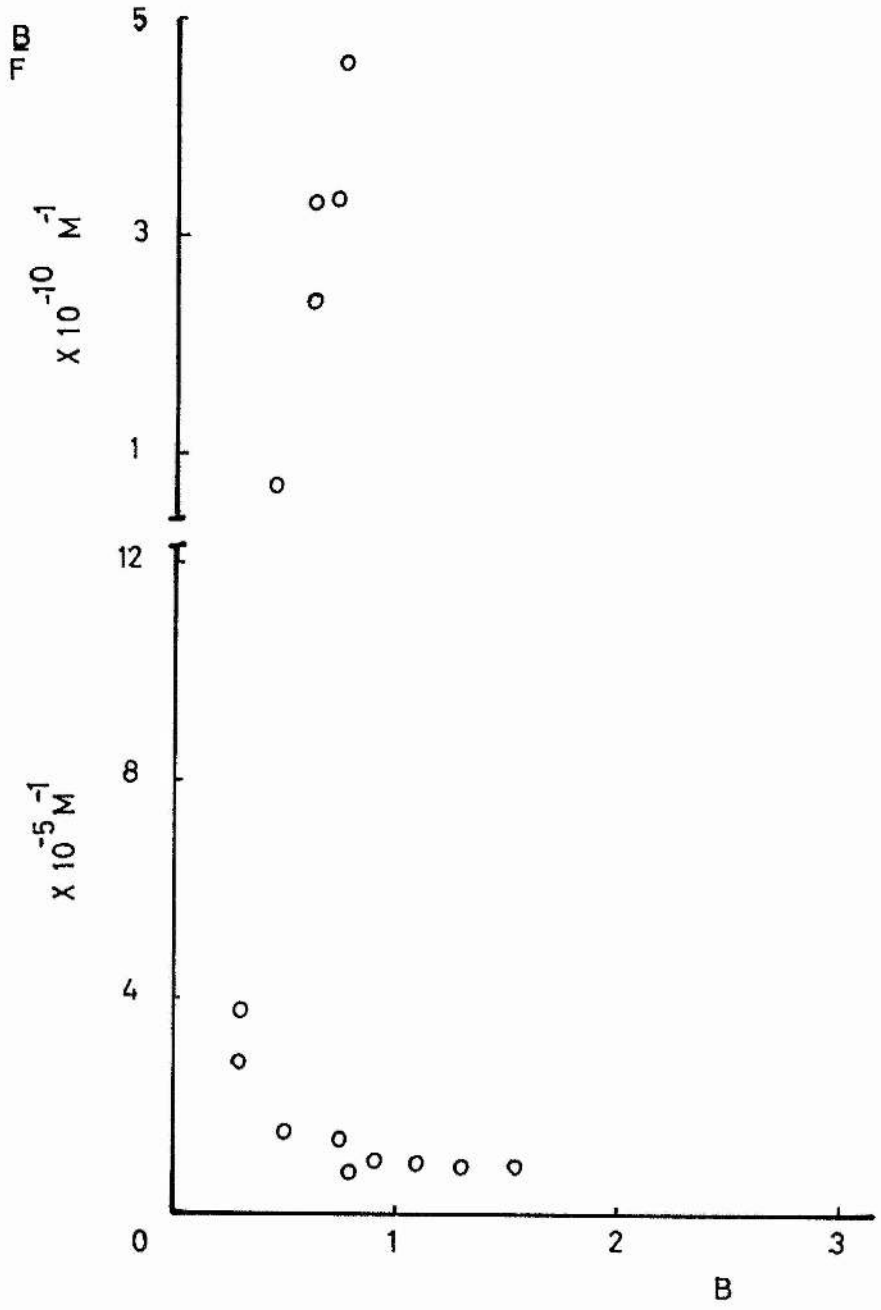
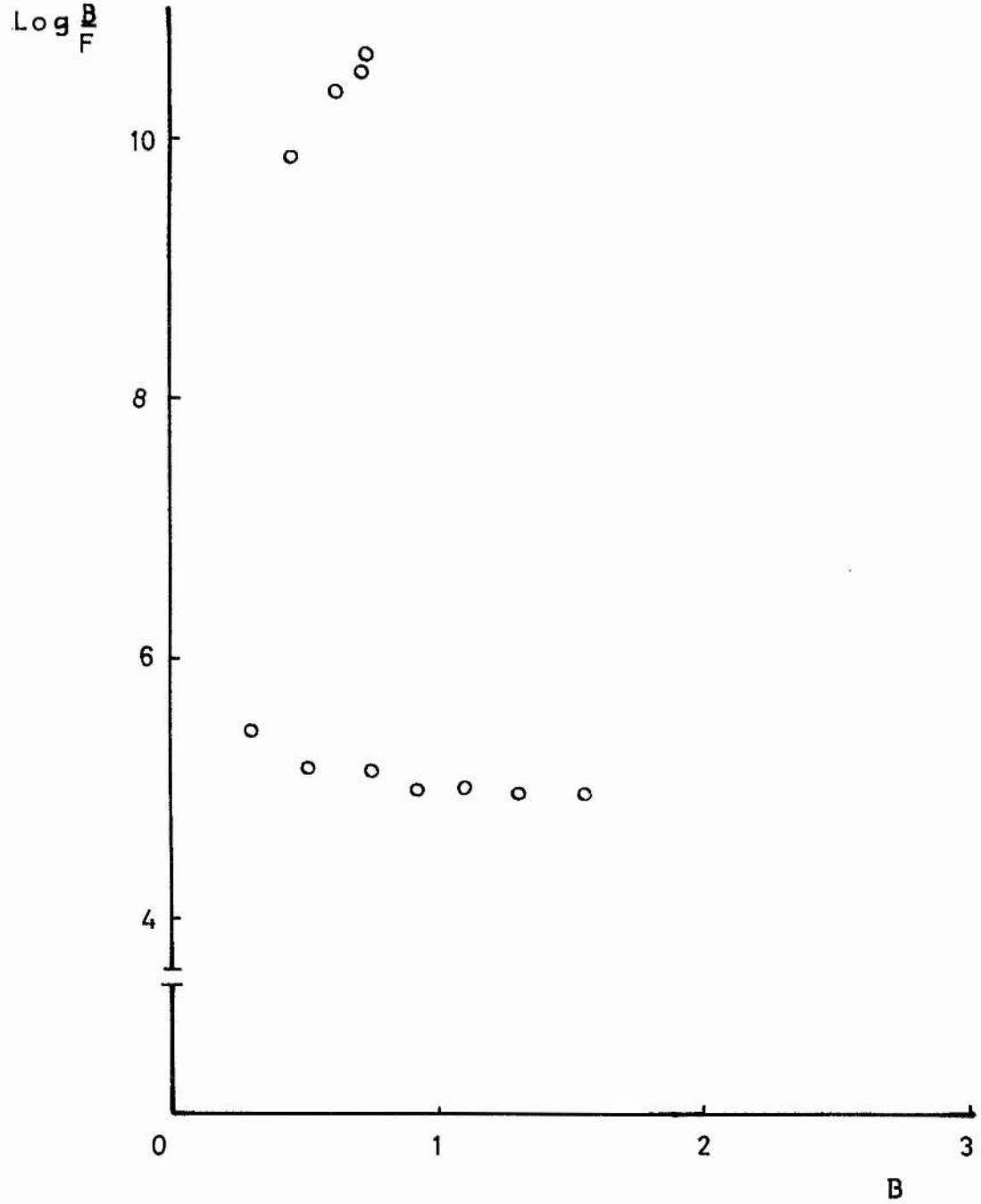

B
F
5
3
1
X 10^-10 M^-1
12
8
4
0
X 10^-5 M^-1
1
2
3
B

Figure 27: Reverse Ligand Binding - In the Presence of 10 mM $MgCl_2$: A Linear-Log Plot of Scatchard Data Obtained from Rate of Dialysis Experiments


Log B/F
10
8
6
4
0
1
2
3
B

sites with different affinities as was done with the results of the ligand binding experiments. Applied to the results for reverse ligand binding, and discounting the B/F values greater than $1x10^{6}M^{-1}$, there are two binding sites $K_d 8x10^{-7}M$ and one site $K_d 7.5x10^{-6}M$. This is at variance with the results from ligand binding experiments and also with published results. However, Scatchard analysis was derived for the interpretation of straight lines rather than curves and there are other possible interpretations of the curves such as co-operativity between calcium binding sites or the binding of chelator to fibrinogen.

DISCUSSION

4.1. PURIFICATION OF FIBRINOGEN

The use of DEAE-Cellulose is a relatively quick and efficient method for improving the quality of Kabi fibrinogen and, in conjunction with Lysine-sepharose, removing proteolytic enzymes which might be present. It is easily adapted to purification of human plasma although could not be carried out at physiological calcium concentrations as, in the presence of 2 mM $CaCl_2$ an insoluble clot formed during dialysis prior to the addition of fibrinogen to DEAE-Cellulose. Even using aluminium hydroxide gel, which removes Factor II (thrombin), Factor VII, Factor IX and Factor X, an insoluble precipitate formed at calcium concentrations of 1 mM and above. When no calcium was added to buffers no significant difference was found during purification between samples prepared in buffer made 0.5 mM with respect to $CaCl_2$ and those prepared in calcium-free buffers. Later work using AAS showed that the calcium concentrations in the fibrinogen samples eluted from DEAE-Cellulose were circa 0.2 mM. It would seem therefore, that there was sufficient contaminating calcium present to maintain a reasonable calcium concentration in the preparations.

The elution profiles from DEAE-Cellulose of Kabi fibrinogen and fibrinogen from outdated blood-bank plasma were similar. Peak I fibrinogen, although not entirely free of A α-chain heterogeneity, is an improvement on commercially available fibrinogen and can be obtained quickly, in under 36 hours, from outdated blood-bank plasma.

Peak II fibrinogen, although degraded, is a good source of fibrinogen for preparations of fragments D and E provided fractions are collected and pooled so that contamination with fibronectin, which is sometimes present in the 'tail' of peak II, is avoided.

The yield of Peak I fibrinogen from plasma varied considerably and was sometimes as low as 50 mg from 100 ml plasma compared to the average fibrinogen concentration in normal plasma of 300 mg/100 ml (Gram, 1922). However, Semeraro et al. (1977) showed that in both commercial fibrinogen (KABI AB) and outdated plasma fibrinogen preparations about 50% of the Aα-chains are degraded. Therefore, if a preparation with a high degree of integrity is prepared a low yield is to be expected. Semeraro et al. concluded that this increased degradation was the result of in vitro degradation during storage. This would partly explain the variation in the yield from different plasmas and the low recovery of Peak I fibrinogen.

The carboxyl terminals of the Aα-chains have been implicated in the high affinity binding of calcium ions. Previous studies of calcium binding in fibrinogen have been made in which human fibrinogen, purified from blood-bank plasma where no extra steps had been taken to ensure a highly intact preparation, was used. Lindsey et al. (1978) used a fibrinogen with a heterogeneous Aα - chain prepared by ammonium sulphate fractionation. Nieuwenhuizen et al. (1979) also used the ammonium sulphate

fractionation and followed this with a gel filtration step on Sepharose 6B. The buffers used throughout the preparation were 3 mM with respect to EDTA. The results of purification of rat fibrinogen by this method (Van Ruijven-Vermeer & Nieuwenhuizen, 1978) indicate a fibrinogen preparation similar to Peak II fibrinogen with some intact Aα-chains and some degraded, detected as a heavy Bβ-chain band when compared to that of the γ-chain on SDS-PAGs. The preparation of a highly intact fibrinogen preparation was considered essential before undertaking any calcium binding experiments.

In order that purification could be completed within 36 h the starting volume from fresh frozen plasma never exceed 180 ml. Preparation of fibrinogen in small quantities had the advantage that storage time was short and therefore the possibility of in vitro degradation of purified fibrinogen was reduced.

4.2. THE HIGH AFFINITY CALCIUM BINDING SITES OF FIBRINOGEN

It has been reported that there are three high affinity calcium binding sites in fibrinogen (Marguerie et al., 1977; Lindsey et al., 1978; Nieuwenhuizen et al., 1979). Two of these have been located in the plasmin digestion fragment D while fragment E has been found not to bind calcium. The third site must, therefore, be present in the remaining parts of the molecule destroyed by digestion by plasmin which are the N-terminal region of the molecule not involved in fragment E, the region of the molecule joining fragment D and E

and the carboxyl half of the Aα-chains. If it is assumed that the symmetry of the fibrinogen molecule is retained it is unlikely that a calcium ion could bridge the two halves of the molecule in the area between fragments D and E irrespective of which model for fibrinogen shape is preferred. The two other possible sites are more compatible with the restraints of a single calcium ion and the maintaining of molecular symmetry. It is possible that a calcium ion is bound in the fibrinopeptide region of the N-DSK and would probably be released by the action of thrombin and during plasmin digestion.However, although efforts to purify N-DSK were successful it could not be obtained in sufficient concentrations at physiological pH because of solubility difficulties. The third possible site for this third calcium ion is in the carboxyl region of the Aα-chain. To maintain symmetry of the molecule this would require overlapping of the Aα-chains with calcium forming a bridge between them. Alternatively the number of high affinity calcium binding sites may have been underestimated. Fibrinogen with a heterogeneous Aα-chain composition has been used previously to obtain calcium-binding data (Lindsey et al., 1978). This heterogeneity arises from degradation at the carboxyl terminals of the chains. If each Aα-chain contained a distinct calcium-binding site using a heterogeneous fibrinogen preparation would give values of between 2 and 4 for the number of calcium ions bound to the molecule.

Marguerie et al. (1977) when working with bovine fibrinogen, found

the third calcium binding site of fibrinogen was destroyed by lowering the pH of the solution to pH 6.0 and suggested that two histidyl residues were involved in chelating the calcium (Figure 28). There are four histidine residues present in the plasmin susceptible area of the N-DSK at Aα -24 and Bβ -16, the precise spacial arrangement of these residues will depend on their relative positions with reference to the disulphide bridges which give the N-terminal of the molecule a high degree of rigidity. The carboxyl terminal of the Aα-chains (610 residues) have each three histidine residues in close proximity at Aα -594, -598 and -604 so that an overlap of the Aα-chains could bring two histidine residues, one from each chain, into close proximity.

There are a large number of calcium-binding proteins catalogued (Kretsinger, 1976) and in the majority of cases the role of calcium is readily explained as either regulating catalytic activity or as a modulator of physiological function. The calcium-binding sites have been sequenced in only a few of these proteins but crystallographic, spectroscopic and chemical studies (Einsphar & Bugg, 1977) have shown that calcium ions preferentially complex with oxygen ligands. In proteins, calcium binds to oxygen atoms on the side chains of glutamic and aspartic acid residues and to the carboxyl oxygen in the peptide backbone. The affinity of calcium for the binding site is determined by the oxygen-calcium distances and the number of oxygen atoms involved.

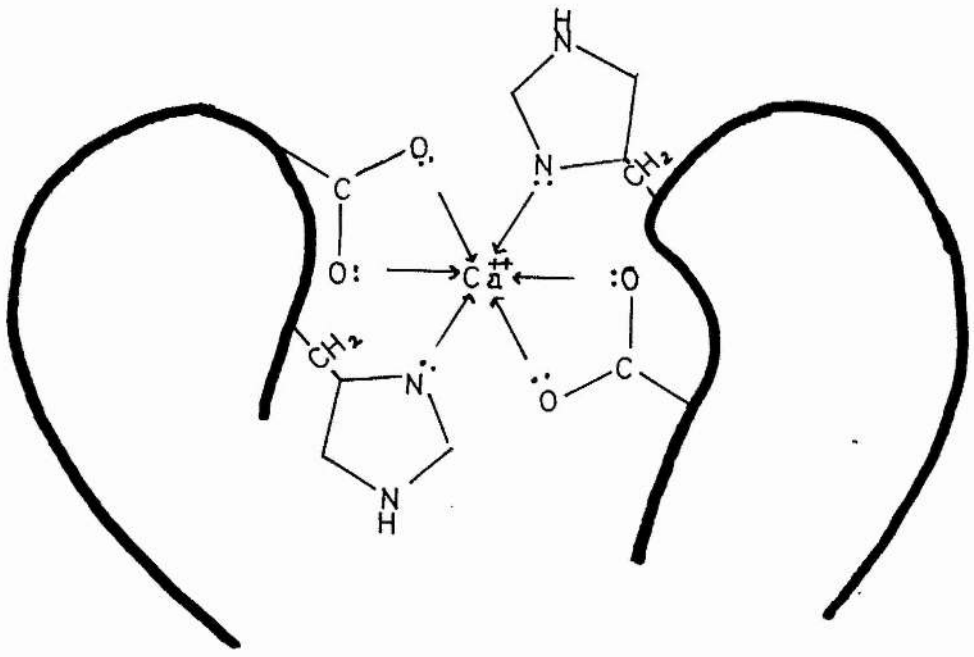


Figure 28: A Model for the Chelation of Calcium Involving Histidine Residues (Marguerie et al., 1977)

The geometry of the oxygen atoms is defined by the tertiary structure of the protein to optimize the metal binding. In the few cases where the proteins have been sequenced the calcium binding sites have been within an intra-chain loop, involving amino acid residues close together in the primary structure of the protein. None of the binding sites so far studied has involved histidyl residues.

A number of the proteins required in blood coagulation bind calcium and most of the coagulation reactions require calcium for optimal function. These calcium dependent reactions involve the vitamin K dependent proteins Factors VII, IX, X and prothrombin. Vitamin K is required for the synthesis of γ-carboxyglutamic acid and this amino acid residue is involved, at least in part, in the metal binding properties of the vitamin K dependent proteins. The γ-carboxyglutamic acid calcium binding sites in prothrombin and Factor X have been studied and found to be homogeneous (Enfield et al., 1975). They have typical calcium binding sites in that calcium binds within an intra-chain loop and spectral studies indicate that a conformational change is induced by the metal ion binding (Benarous & Gacon, 1980; Lindhout & Hemker, 1978). However the γ-carboxyglutamic acid binding sites have dissociation constants of 10^{-3}-10^{-4} M (Nemerson & Furie, 1980), an order of magnitude higher than the values quoted for the calcium binding sites in fibrinogen. Also, no γ-carboxyglutamic acid residues have, as yet, been identified in fibrinogen.

Fibrinogen was cross-linked with the bifunctional agent dimethyl suberimidate dihydrochloride, which reacts with the free amino groups of proteins, as a means of investigating conformational differences which might occur in the presence and absence of calcium. There was no apparent change in the electrophoretic pattern obtained from fibrinogen cross-linked in the presence of calcium and in the presence of EGTA. Nor was there any evidence, as determined by N-terminal analysis, of an $A\alpha$ -$A\alpha$ dimer formed by cross-linking. These results, taken in conjunction with previous work (Ross & Kemp, 1979) where cross-linked fibrinogen, prepared in phosphate buffer and digested with cyanogen bromide, gave a digestion pattern indistinguishable from that of non-crosslinked fibrinogen, suggests that cross-linking occurs predominantly within the fibrinogen half-molecule rather than linking the two halves of the molecule. Furlan & Beck (1975) found a similar pattern of cross-linking with gluteraldehyde which is thought to react with ε-amino groups of lysine residues. The intra-molecular cross-linking occured predominantly between β-γ and α-β chains within the N-DSK.

Radioactive labelling with ^{45}Ca was used to investigate the high affinity calcium binding sites of fibrinogen. The work of Lawrie & Kemp (1979) implied that calcium ions remained in situ within the binding site in fibrinogen during electrophoresis. However, using radioactively labelled calcium this was not confirmed.

The presence of a high mobility γ - chain is probably the result of the stabilisation of the disulphide bond at the carboxyl terminal of the γ - chain under mild denaturing conditions rather than a direct effect of the binding of calcium to the γ - chain.

The first region of the fibrinogen molecule to be attacked by plasmin is the carboxyl terminal of the Aα-chain (Marguerie et al., 1977). If a calcium ion binding site is located in this region of fibrinogen a limited digestion by plasmin should result in a reduction of the binding capacity of the molecule. Initial experiments, in which a ^{45}Ca-fibrinogen sample was digested and the concentration of bound calcium compared with that in a non-digested sample gave encouraging results. However, these proved to be misleading as the conditions under which the samples were prepared were not sufficiently stringent as later work showed. The buffers used in the preparations had a high concentration of contaminating calcium and dialysis against EDTA is not sufficient to give a calcium free preparation. Thus the ratio of ^{45}Ca-ions to ^{40}Ca-ions was overestimated. It is also doubtful that the desalting technique used was sufficiently effective in removing all but the tightly bound calcium from solution. The experiment was repeated using the flow dialysis apparatus. A calcium ion bridging the two Aα-chains would be expected to be released into the media at a very early stage of plasmin digestion and, even assuming the chelate complex retains the calcium ion, the resulting polypeptide chain should be small enough to pass through a dialysis membrane. Figure 20 shows

the result of such a digestion followed by the flow dialysis technique. 85% of the calcium is free therefore most of the high affinity calcium binding sites are occupied as the total calcium concentration was 5.75×10^{-4} M and the protein concentrations 1.75×10^{-5} M. Despite this and degradation of the Aα-chain to a molecular weight of less than 56,000 no calcium was seen to be released. These results indicate that the carboxyl terminal regions of the Aα-chains are not involved in calcium-binding. This is in agreement with work recently published by Nieuwenhuizen & Gravesen (1981). They found no significant differences in the calcium binding properties of fibrinogen and plasmin fragment X.

4.3. PREPARATION OF CALCIUM-FREE SOLUTIONS

Buffers prepared from standard laboratory chemicals were found to contain trace amounts of calcium when measured by Atomic Absorption Spectrometry. The primary source of contamination was NaCl and from this calcium was quickly removed by use of Chelex-100 resin. Buffer prepared from Tris (BDH) and Trizma (Sigma) were never satisfactorily freed from calcium and it would seem that this base has a significant affinity for calcium. This problem was not apparent with imidazole buffer which did not require Chelex-100 treatment.

$MgCl_2$ is frequently included in buffer systems when measuring the binding of calcium to fibrinogen as it eliminates the low

affinity calcium binding sites (Marguerie et al., 1977). It is also contaminated with trace amounts of calcium, which are significant as $MgCl_2$ is added at concentrations of 10 mM, and this cannot be removed by chelating resins as their affinity for calcium and magnesium ions are similar. However if fibrinogen samples with suitably low calcium concentrations could be prepared, two ligand binding experiments could be performed to obtain a full spectrum of values for a Scatchard plot. In this way magnesium-free buffers, could be used to assure low calcium concentrations when small bound values, at low calcium concentrations, were being measured and magnesium containing buffers employed to eliminate low affinity calcium binding sites at higher calcium concentrations.

There appear to be two main criteria which affect the final calcium concentration of 'calcium-free' fibrinogen preparations; the length of dialysis and the degree of contamination of the dialysis buffers. Pre-treatment of dialysis tubing, either by extensive treatment to remove calcium ions or by acetylation, did not have any significant bearing on the final calcium concentrations of the fibrinogen solutions. The concentrations of the trace amounts of calcium in buffer solutions is the limit of efficiency of the dialysis procedure. The use of Chelex-100 treated buffers was, therefore, essential. The results of long dialysis showed an apparent increase in the calcium concentration of the sample after the initial decrease brought about by EGTA. The reason for this is obscure. It is possible that calcium ions, which are known to bind to cellulose membranes, are released slowly into the media but this is difficult to envisage as it would require μmoles

of calcium to be released from the dialysis tubing.

Before dialysis to remove calcium, fibrinogen was freeze-dried so that a final solution of higher concentration (5-9 mg/ml) could be obtained. This method is not ideal as it has been known to cause inactivation of enzymes and in some cases degradation of proteins. The latter point is of particular importance in this study of fibrinogen. However, provided lyophylisation was carried out for as short a time as possible no differences in molecular weight were found between fibrinogen before and after freeze-drying when examined by 10% SDS-PAGE. Freeze-drying was preferred to ultrafiltration methods, such as Amicon Ultrafiltration Cells or Unicam Millipore Immersible Separator Kits, for concentrating protein solutions. These methods, which employ filtration under pressure through a membrane, were found to give poor recovery of fibrinogen with around 25% and sometimes as much as 50% being lost. Most of the loss could be accounted for by fibrinogen adhering to membrane and vessel surfaces and this fibrinogen was difficult to remove without redilution of the protein solution.

4.4. FLOW DIALYSIS

4.4.1. Preliminary Experiments

The results from experiments using a double layer of Sigma or Visking tubing or the dialysis sheets illustrate the artefacts, highlighted by Reed (1973), which arise from the binding

of calcium ions to unmodified cellulose membranes. The cellulose membranes appear to have a large number of low affinity calcium binding sites the effects of which can be almost completely eliminated by the presence of other cations, such as sodium or potassium, in the dialysis buffer. This is demonstrated by the results from single layer Sigma and Visking membrane experiments which, as in all the preliminary experiments, were performed in the presence of NaCl. On the addition of ^{40}Ca-ions to the top chamber there was no change in the steady-state level of radioactivity in the eluent. This was not the case when double layer membranes or the dialysis sheets were used. In these experiments the appearance of a peak of radioactivity in the eluent followed the addition of ^{40}Ca-ions to the upper compartment. This suggested, along with the slow equilibration of the system, that calcium ions were binding to the surface of the cellulose and perhaps also within the cellulose matrix. This latter option may be of considerable significance with regard to the dialysis sheet as it is considerably thicker than the other cellulose membranes tested.

A more reliable method of eliminating the binding sites is by acetylation of the membrane and this had the added benefit of reducing slightly the loss of calcium across the membrane. However acetylation did not reduce the equilibration volume

required with Sigma and Visking double membranes as would have been expected. This suggests that the slow attainment of the plateau level of radioactivity in the eluent is not due to calcium binding to the membrane but is a result of compartmentation, with a film of solvent trapped between the two layers of membrane giving rise to two different concentration gradients, which will change the equilibration characteristics of the system. However with single layer acetylated Sigma and Visking membranes the rate of dialysis, and hence the concentration of isotope in the eluent, is dependent only on the fraction of the ligand free in the upper chamber and not the total concentration of the ligand.

The flow dialysis cell was designed for the rapid removal of small molecules and is therefore not ideal for studying ion/protein binding as it has a large membrane surface. In other studies where this type of cell has been used (Colowick & Womack, 1969; Hegyvary & Post, 1971) the labelled moiety has been a substrate or cofactor rather than an ion. Thus binding of the labelled ligand to the membrane has not been of great significance and a more efficient membrane, with respect to the retention of the radioactivity in the upper compartment, has been used without any of the artefacts, caused by the binding of the labelled ligand to the membrane, arising.

For accurate quantitative data to be obtained from flow dialysis experiments, theoretically, no label should be lost from the

upper chamber. Practically, this loss must be minimised and previous workers have found that a total loss of up to 5% can be accommodated. The modification of the flow dialysis cell by the addition of an insert which reduced the surface area of the membrane available for dialysis markedly decreased the level of radioactivity in the eluent without the introduction of any artefacts. This modification made possible the use of the flow cell for rate of dialysis experiments.

4.4.2 Ligand Binding

Norby et al. (1980) have criticised the widespread misinterpretation of ligand-protein binding experiments which show upward curvature in Scatchard Plots. Their two principle complaints were that the upward curvature was always associated with, and interpreted as, multiple classes of independent binding sites with little consideration given to other possible interpretations such as negative co-operativity and the misinterpretation of these plots when calculating the binding constants and number of binding sites (Figure 29). Until recently none of the groups active in the determination of calcium-fibrinogen binding data had publicly re-assessed their data although recently Nieuwenhuizen et al. (1981b) have done so. In addition calcium binding studies in fibrinogen have been carried out using heterogeneous fibrinogen preparations. In view of this it was decided to study high affinity calcium-fibrinogen binding with the technique of flow dialysis a method by which data can be obtained quickly

thereby minimising any degradation which might take place during the course of the experiment.

Many problems were encountered with this technique, some being unique to this particular study. Calcium is a common contaminant in many laboratory reagents and is not, as has been discussed previously, always easily removed. Also, calcium ions, because of their size, have a high rate of dialysis and this results in a rapid loss of label from the reaction chamber. Even the use of acetylated membranes and reduction of the exposed membrane surface area does not reduce the loss to levels which can be ignored for the purpose of calculation of results. Yue & Gertler (1978) studied calcium binding to bovine Factor X and encountered similar problems with a 20% loss of label throughout the duration of the experiment although this may be corrected for in the final results.

The difficulty in obtaining calcium-free fibrinogen solutions has more bearing in flow dialysis than in equilibrium dialysis as in the former it is the initial calcium concentration of the protein solution which is of importance whereas in equilibrium dialysis it is the final calcium concentration after dialysis which is measured. The calcium concentration is the sum of the residual amount of calcium present in the fibrinogen preparation and the calcium concentration of the $^{45}CaCl_2$ solution. The concentration of the latter is comparatively high

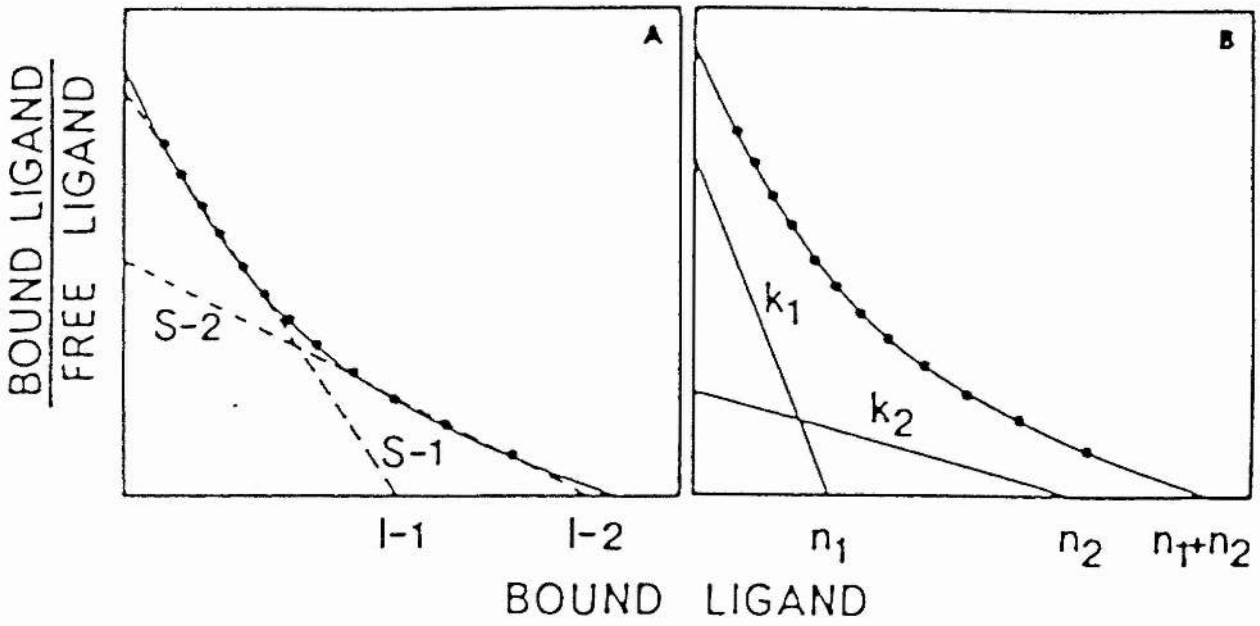


Figure 29: The Interpretation of Scatchard Plots

A) Mis-interpretation. The gradients of S-1 and S-2 will not give true association constants

B) True association constants can be obtained from K_1 and K_2

(Norby et. al., 1980)

as the activity must be sufficient for counting purposes when only a negligible quantity of label is lost to the effluent. This is particularly important if efforts have been made to decrease the percentage of label leaving the upper chamber. Users of equilibrium dialysis may also employ Ca/EGTA buffers as was done by Marguerie et al. (1977), to give low free calcium concentrations, a method which is not viable with flow dialysis, although the use of ^{40}Ca/EGTA buffers in calcium protein binding studies has been criticised by Wikman-Coffeld & Muhlrad (1980) who state that isotope dilution procedures using such a system cannot be used to accurately determine the molar ratio of ligand to protein. Another problem was fibrinogen itself. With a molecular weight of 340,000 a solution of 5 mg/ml has a relatively low concentration of binding sites.

These factors combined resulted in a loss of sensitivity of the technique and measurements at bound values of less than 1 were not possible. Results from ligand binding experiments demonstrated the presence of three equivalent high affinity calcium binding sites which is in agreement with the work of Lindsey et al. (1978) although there is a discrepancy, by a factor of two, between the calculated binding constants. Their value was also derived from data in which there were no bound values between 0 and 1. Nieuwenhuizen et al. (1979) obtained different results.

Their bound values encompassed a wide range between 0 and 3 and the resulting Scatchard plot was a curve. By drawing tangents to the curve two classes of binding site were differentiated. However this data has recently been re-assessed (Nieuwenhuizen et al.,1981b) and they now propose three equivalent calcium binding sites with K_d1.9x10^{-5} M a value similar to that obtained by flow dialysis experiments.

The conformation of three high affinity calcium binding sites and the elimination of the carboxyl regions of the A -chains of having involvement in calcium binding leaves the location of the third site undetermined. It would be of interest to know the role of this third binding site. If it has a role in the first steps of fibrin polymerisation it may be within the N-DSK in the N-terminal regions not present in Fragment E.

4.4.3. Reverse Ligand Binding

In order to obtain better quantative data the method of Haeich et al. (1980), requiring additions of EGTA to a constant calcium ion concentration was employed. This method is preferable to the use of EGTA/Ca buffers although errors introduced by differences in affinity of $^{40}Ca^{2+}$ ions and $^{45}Ca^{2+}$ ions by EGTA (Nash et al., 1979) or by the binding of the chelator to protein may still arise (Haiech et al., 1979). In the absence of $MgCl_2$ the resulting Scatchard plot had a very high upward curve at the bound value of circa 1 with some suggestion of the curve falling back on itself.

If $MgCl_2$ was included in the buffers the effect was accentuated. The presence of magnesium ions also caused a shift of the curve to the left because the presence of these ions in the system was not taken into account in the calculation of bound and free values. Magnesium ions will bind to EGTA therefore, the results shown (Figure 26) assume a higher EGTA concentration than was actually available for chelating calcium.

Although EGTA has a much higher binding affinity for calcium than magnesium, 10^{10} M^{-1} compared with $10^{5}M^{-1}$, the high magnesium concentration of 10 mM will affect the calcium-EGTA equilibrium. Magnesium will remove EGTA from the system and as a result the EGTA-calcium equilibrium will shift towards more free, i.e. unchelated, calcium. This in turn will mean a small increase in the protein bound calcium. Thus for a given set of chelator concentrations, in the presence of magnesium ions, the concentration of free (unchelated) calcium is underestimated as is the concentration of protein-bound calcium. As the numerical value of the free calcium is underestimated to a greater degree than the bound value, at every bound value the value of bound/free will be overestimated. Hence the resulting graph, when compared to a similar result obtained from experiments performed in the absence of magnesium, will have shifted to the left.

The curves from reverse ligand binding experiments do not resemble the Scatchard plot obtained by Niewenhuizen et al. (1979) or that from ligand binding (Figure 22) with the primary difference the

severe upward curvature not present in the previous studies. A number of possible explanations for this shape of graph must be considered.

1) Reverse ligand binding requires the use of chelating agents, in this case EGTA and assumes that EGTA has an equal affinity for ^{40}Ca and ^{45}Ca. But this assumption is untrue as the binding constant of ^{45}Ca/EGTA is greater that that of ^{40}Ca/EGTA (Nash et.al., 1979). Wikman-Coffelt & Muhlrad (1980), working with myosin and using tracer techniques , concluded that the binding affinity values were similar to those obtained by other methods which did not involve using ^{45}Ca. Therefore the effect of difference in binding affinities of EGTA for ^{40}Ca and ^{45}Ca would appear to be negligible.

2) Co-operativity of sites must also be examined. Positive co-operativity yields a convex plot with with a maximum (McGhee & Van Hippel, 1974)(Figure 30) while negative co-operativity gives a concave curvature (Thakur et al., 1980), similar to that obtained for different independent sets of equivalent binding sites (Weder et al., 1974). It is difficult to imagine a function for co-operativity between binding sites in fibrinogen as the only known function of the calcium ion bound in the Fragment D region of the molecule is as a protector against

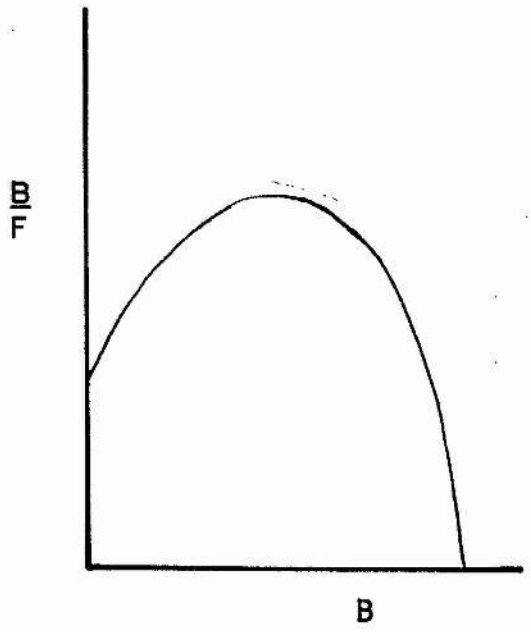


Figure 30: Scatchard Plot of a System Demonstrating Co-operative Binding (McGhee & Von Hippel 1974)

plasmin digestion (Haverkate & Timan, 1977) with perhaps some role in the cross-linking of fibrin monomers by Factor XIII. Nor is it likely that if there was co-operativity between sites it would result in such a dramatic change in the value of bound/free.

3) Another possible interpretation of the concave curve is that there is more than one set of independent binding sites. There is little evidence that there are, in fibrinogen, calcium binding sites with greater affinity than the three high affinity binding sites previously discussed. When Nieuwenhuizen et al. (1981b) re-assessed their data for the calcium binding sites in human fibrinogen the computer fit of the data improved substantially when 0.08 sites with $K_d 3 \times 10^{-7}$ M were allowed for in the Scatchard plot. But Marguerie et al. (1977) used Ca/EGTA buffers when investigating the binding sites in bovine fibrinogen and found no calcium binding to fibrinogen at calcium concentrations between 1×10^{-8} M and 5×10^{-7}M.

4) Haeich et al. (1980) warned that using EGTA could lead to anomalies, as some proteins are known to bind this chelating agent, but gave no indication as to how this would affect a Scatchard plot of the data. If the Scatchard plot obtained by Niewenhuizen et al. (1979) (Figure 31) is examined it can be seen that the point

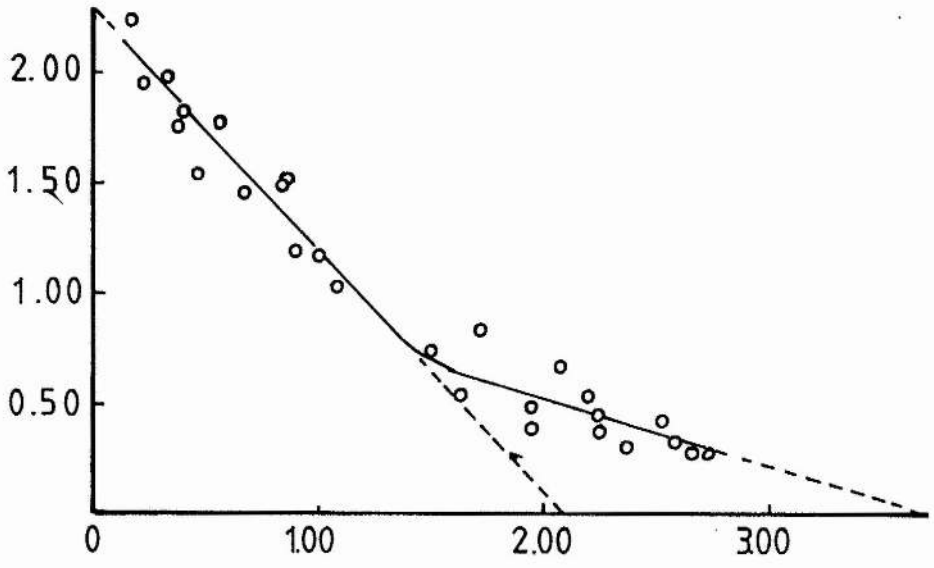


Figure 31: Scatchard Plot of Number of Calciums Bound/mol Human Fibrinogen and Free Calcium Concentration

Nieuwenhuizen et. al. (1979)

of inflection of the curve is bound ~ 1.5. If the Scatchard plots of reverse ligand binding in the absence (Figure 24) and presence (Figure 26) of $MgCl_2$ are examined the points of inflection occur at bound ~ 1.25 and bound ~ 0.5 respectively. Deranleau (1969) has shown that for low and high saturation of sites the error in the Scatchard plot is disproportionately larger than in the range of half-saturation. A closer scrutiny of the treatment of the data gives an indication of which factors influence the final value of B/F (Table VIII)

Free ligand (L) is calculated from equation

$$(L) = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a} \quad .. \; .. \; .. \; .. \; (4)$$

To obtain a positive value for (L) the positive root was used and as 'a' contained no variables '2a' was always constant so the variable part of the equation is

$$-b + \sqrt{b^2 - 4ac} \quad .. \; .. \; .. \; .. \; .. \; (6)$$

4ac is numerically much smaller that b^2 therefore the numerical values of -b and $\sqrt{b^2 - 4ac}$ are very similar. The large increase in B/F coincides with a change in the sign of 'b' from negative to positive as the value of statement (6) changes dramatically from ~ 2b to ~0. Thus the value of (L) tends to be 0 and, as a result, B/F tends to infinity, (Table VIII).

$[^{45}Ca]$ in eluent ($\times 10^{9}$ M)	[EGTA] upper chamber ($\times 10^{6}$ M)	'b' ($\times 10^{9}$ M)	'c' ($\times 10^{20}$ M)	$\sqrt{b^2 - 4ac}$ ($\times 10^{9}$)	(L)
.467	50	-0.21	-0.47	0.21	0.11×10^{-4}
.440	60	-0.13	-0.44	0.13	0.67×10^{-5}
.408	70	-0.044	-0.41	0.044	0.23×10^{-5}
.437	80	-0.021	-0.44	0.021	0.11×10^{-5}
.434	90	+0.034	-0.43	0.034	0.12×10^{-9}
.430	100	+0.090	-0.43	0.090	0.47×10^{-10}

Table VIII: Calculation of (L). The Influence of Intermediate Factors on the Value of (L).

This change from negative to positive value of 'b' explains the dramatic jump from 10^5 M^{-1} to 10^{10} M^{-1} in the values of B/F and at such values the suggestion of the graph folding back on itself can be attributed to error.

The value of 'b' is calculated from

$$C_1C_3 + C_2V_2 - V_1 \quad .. \quad .. \quad .. \quad .. \quad (5)$$

where V_1 = concentration of ^{45}Ca in the effluent

V_2 = concentration of EGTA in upper chamber

At low EGTA concentrations $\overline{V}_1$ is greater than $\overline{C_1C_3 + C_2V_2}$, as the concentration of EGTA increases $\overline{V}_1$ suddenly becomes less than $\overline{C_1C_3 + C_2V_2}$. During flow dialysis a small percentage of label is lost throughout the experiment and towards the end of the experiment this error is larger. This implies that the measured value of V_1 is smaller than the 'perfect' experiment where no calcium is lost from the upper chamber. The same effect could be brought about by the overestimation of V_2 because of the presence of magnesium ions or the binding of EGTA to the fibrinogen molecule. As the loss of calcium from the top chamber was very small, abour 3% of the total, and because of the indirect evidence suggested by the buffers containing magnesium it would seem reasonable to suggest that EGTA binds to fibrinogen.

The effect of chelating agents on fibrinogen is not well

documented. In studies using EDTA, Godal (1960,1969) concluded that all the effects of the chelating agent, such as loss of heat resistance and prolonged thrombin time, could be attributed to its ability to chelate calcium. Bithell (1964) investigated in more detail its effect on the thrombin clotting time and again concluded that the results could be explained by the chelating properties of EDTA. He also dialysed EDTA out of the fibrinogen solution in the presence of 0.15 M NaCl but the absence of divalent cations and on the return to near-normal thrombin clotting time concluded that EDTA reacted directly with fibrinogen. Blomback et al. (1966) examined the effect of EDTA on the sedimentation rate of fibrinogen and again found the results indecisive. The development of better methods for detecting trace amounts of divalent cations has cast further doubts on these results. Very recently Nieuwenhuizen et al (1981a) using equilibrium dialysis, investigated EDTA binding to fibrinogen and concluded that 0.4 moles EDTA bound to each mole of fibrinogen with K_d 2.2 x 10^{-5} M.

The results presented here suggest that another, related, chelating agent, EGTA, also binds to the fibrinogen molecule.

Most previous studies of the role of calcium in fibrinogen have involved comparative studies in the presence of EDTA/EGTA and in the presence of calcium. Although only a small amount of chelator binds it may be that some of the functions

which are lost when calcium is removed and, therefore, attributed to the presence of calcium ions, may be inhibited by the chelating agent binding to the fibrinogen molecule.

In conclusion, the results of this study show that -

1. The high affinity binding of calcium to fibrinogen does not involve the carboxyl terminal regions of the Aα -chains.

2. The use of a highly intact fibrinogen preparation does not alter the published values of three high affinity calcium binding sites.

3. There is indirect evidence which suggests that the chelating agent EGTA binds to the fibrinogen molecule.

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COMPUTER ANALYSIS OF DATA FROM LIGAND BINDING AND REVERSE LIGAND BINDING EXPERIMENTS

The intuitive interpretations from analyses of Scatchard data have been criticised (Norby et al., 1980). However, it was only after the completion of this work that the opportunity arose to analyse the data obtained from ligand binding and reverse ligand binding experiments using a non-linear, model-fitting programme.

The programme used was "Ligand" developed by Munson & Rodbard (1980). This programme is a multi-purpose computer system for the analysis of ligand binding data, model fitting and parameter estimations. It can be used to find the most appropriate model for the data as well as the best parameter estimates for that model. As with all non-linear curve-fitting programmes, the initial estimates for all the parameters must be provided by the operator. These may be made from graphical analyses of Scatchard data. If a number of different models are tried the programme will give the best representation of the data and the best parameter estimations for that model.

Results

Data from both ligand binding and reverse ligand binding experiments were subjected to computer analysis.

Ligand Binding: The data was found to fit two models with equal probability.

If one class of binding site was assumed the data was consistent with 3.03 binding sites of K_d 1.2 x 10^{-5}M. This gives good agreement with the values obtained by the inspection of graphic analysis of three sites of K_d 1.4 x 10^{-5}M. If two classes of binding sites were assumed the resulting parameters were -

0.98 binding sites of K_d 1.5 x 10^{-6}M

1.88 binding sites of K_d 2.4 x 10^{-5}M

Both the one class of binding site and the two classes of binding site models are equally compatible with the given data. As noted previously, however, the data does not cover an extensive range of "bound" values. Hence the data points available may correspond to the central part of a curve which can be interpreted as a straight line. Consequently, in order to establish which model best fits the Ca^{2+}-fibrinogen system, more values at both high and low calcium concentrations are required. However if data obtained at high calcium concentrations is included other parameters, relating to the low affinity calcium-binding sites ($K_d \sim 10^{-3}$M), must be taken into consideration unless magnesium ions have been included in the dialysis buffers.

Reverse Ligand Binding: Only data obtained from experiments in which magnesium ions had been omitted from buffer solutions were successfully analysed. Scatchard plots of data obtained from experiments in which

magnesium buffers had been used showed a marked backward curvature and no model could be found which gave a good fit for the data.

Using all data points, the "best-fit" model was obtained when two clases of binding site were assumed giving -

2.5 binding sites of K_d 3.7×10^{-5}M

1.1 binding sites of $K_d \leq 10^{-7}$M

The dissociation constant for the higher affinity site varied between 10^{-7} and 10^{-10}M with different sets of data. This variability is in part due to the inherent error in the data points corresponding to low calcium concentrations obtained towards the end of the flow dialysis experiment. However, there is a significant difference between the results of ligand binding and reverse ligand binding experiments. If EGTA binds to fibrinogen this will affect the value of the bound and free values as described previously. In addition EGTA may directly affect the binding of calcium to fibrinogen by binding at or near a calcium-binding site. Alternatively EGTA could itself bind calcium while binding to the fibrinogen molecule. Both these possibilities could explain the high affinity binding site $K_d \leq 10^{-7}$M,

indicated by reverse ligand binding experiments.

Conclusions

1. Computer analysis of ligand binding data supports the model for three equivalent calcium-binding sites in fibrinogen. It also finds a model of three calcium ions binding at two classes of binding site equally compatible with the presented data.

2. Data from reverse ligand binding experiments give a different "best-fit" model than that obtained from ligand binding experiments. Thus implying some direct effect by EGTA on the fibrinogen-calcium system.

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